WWW.EJONCOLOGY.IT

MATTIOLI 1885

WWW.MATTIOLI1885.COM

Eur. J. Oncol. - Vol. 23 - N. 2 - June 2018 | ISSN 1128-6598

EUROPEAN JOURNAL OF ONCOLOGY

The European Journal of Oncology is indexed by Excerpta Medica (EMBASE), the Elsevier BioBASE , Scopus (Elsevier) and Bibliovigilance

MATTIOLI 1885

Pubblicazione trimestrale - Poste Italiane s.p.a. - Sped. in A.P.- D.L. 353/2003 (conv. in L. 27/02/2004 n. 46) art.1, comma 1, DCB Parma - Finito di stampare Novembre 2018

RAMAZZINI INSTITUTE

EUROPEAN JOURNAL OF ONCOLOGY

free on-line: www.ejoncology.it

Founded by: Leonardo Caldarola, Cesare Maltoni

Editors

managing editor
Morando Soffritti (Bologna)
executive editors
Francesco Di Costanzo (Firenze)
Paolo Pronzato (Genova)

associated editors
Fiorella Belpoggi (Bologna)
Francesco Cognetti (Roma)
Pietro Comba (Roma)
Angela Guaragna (Bologna)
Evaristo Maiello (S. Giovanni Rotondo)
Beniamino Palmieri (Modena)
Armando Santoro (Rozzano)
Mario Taffurelli (Bologna)
Edoardo Triggiani (Bari)

scientific secretariat
Lorenzo Antonuzzo (Firenze)
Elisa Giommoni (Firenze)
Michela Padovani (Bologna)

editorial board
Arcangeli Annarosa (Firenze)
Ardizzoni Andrea (Parma)
Ascierto Paolo (Napoli)
Benedetti Panici Pierluigi (Roma)
Biasco Guido (Bologna)
Bochicchio Francesco (Roma)
Boggi Ugo (Pisa)
Boni Corrado (Reggio Emilia)
Boni Luca (Firenze)
Brandes Alba (Bologna)
Brazier Jill V. (Bologna)
Cartenì Giacomo (Napoli)
Casali Paolo (Milano)
Cascinu Stefano (Ancona)
Ciardiello Fortunato (Napoli)
Conte Pier Franco (Padova)
Coppola Roberto (Roma)
Crinò Lucio (Perugia)
Crosignani Paolo (Milano)
Cuzzocrea Diego Ettore (Bologna)
Dal Mastro Lucia (Genova)
Danova Marco (Pavia)
De Braud Filippo (Milano)
Degli Esposti Davide (Lione, France)
Di Leo Angelo (Prato)
Englund Anders (Solna, Sweeden)
Falcone Alfredo (Pisa)
Fazio Nicola (Milano)
Forastiere Francesco (Roma)
Garassino Marina (Milano)
Gennaro Valerio (Genova)
Gianni Luca (Milano)
Gori Stefania (Negrar)
Graziano Francesco (Pesaro)
Huff James (Research Triangle Park, USA)
Lambertini Luca (New York, USA)
Landrigan Philip J. (New York, USA)
Lelli Giorgio (Ferrara)
Licitra Lisa (Milano)
Liu Zhuoming (Cleveland, OH, USA)
Maio Michele (Siena)
Mehlman Myron (Princeton, USA)
Messerini Luca (Firenze)
Mini Enrico (Firenze)
Mussa Antonio (Torino)
Normanno Nicola (Napoli)
Paolucci Paolo (Modena)
Paradiso Angelo (Bari)
Parkin Max (Oxford, UK)
Passalacqua Rodolfo (Cremona)
Pileri Stefano (Bologna)
Pinto Carmine (Parma)
Pizza Giancarlo (Bologna)
Puglisi Fabio (Milano)
Sammarco Giuseppe (Catanzaro)
Sanguinetti Giuseppe (Roma)
Santini Donatella (Bologna)
Saxe Einbond Linda (New York, NY, USA)
Scagliotti Giorgio (Torino)
Siena Salvatore (Milano)
Solli Piergiorgio (Milano)
Sternberg Cora (Roma)
Taffurelli Mario (Bologna)
Tortora Giampaolo (Verona)
Trodella Lucio (Roma)
Valentini Vincenzo (Roma)
Valeri Andrea (Firenze)
Verusio Claudio (Saronno)
Viale Giuseppe (Milano)
Zagonel Vittorina (Padova)
Zaridze David (Moscow, Russia)

editorial staff
ramazzini institute (bologna)
Erica Tommasini (Head Editor)
Luciano Bua
Annalisa Buscaroli
Marco Manservigi
Fabiana Manservisi
Isabella Manzoli
Rita Montella
Simona Panzacchi
Federica Scagliarini
Valentina Strollo
Eva Tibaldi

Mattioli 1885
srl | strada della lodesana, 649/sx, loc. vaio
43036 fidenza (pr), italy
tel. ++39 0524 530383
fax ++39 0524 82537
e-mail: redazione@mattioli1885.com
www.mattiolihealth.com

The European Journal of Oncology (Eur. J. Oncol.) publishes Original Articles, Commentaries, Review Articles, Case Reports of clinical and traslational oncology. The manuscript must be submitted using the journal web site:
www.ejoncology.it
The Editorial Office will forward the manuscripts to the Editors-in-Chief, Prof. F. Di Costanzo, Prof. P. Pronzato, Dr. M. Soffritti,
For any information please refer to:
European Journal of Oncology – Publisher Editorial Office
Dr. Valeria Ceci - Mattioli 1885 srl
Strada di Lodesana 649/sx, Loc. Vaio - 43036 Fidenza (PR) - Italy
E-mail: publisher@ejoncology.it - Fax: 0039-(0)524-82537

The Journal does not hold itself responsible for statements made by contributors or for loss or damage of mailed manuscripts. They should be accompanied by an undertaking that they are submitted to this Journal only. Papers must be submitted in English. Papers are accepted on the understanding that they may be subject to editorial revision.
All Original Articles are subject to review and authors are urged to be brief. Long papers with many tables and figures may require shortening if they are to be accepted for publication. All manuscripts should include a total text word count and an abstract word count on the cover page. Total text word count does not include title page, figure legends, references, or tables. Only under exceptional circumstances will Original Articles longer than 5500 words be considered, and under no circumstances will abstracts greater than 250 words be published. Editorials and Reviews are normally invited contributions but suitable papers may be submitted to the Editor for consideration for this purpose. The presentation of Case Reports should be as short as possible. Letters to the Editor should not exceed 600 words of text, one figure or table and up to six references. Because space limitation, publication of submitted Letters will depend on priority rating.

TITLE PAGE must contain:
- a concise informative title
- author(s) names
- department or institution where work was done
- name and address of author to whom correspondence about the manuscript and request for reprints should be referred, as well as fax, E-mail and telephone number
- a running title of no more than 40 characters.

Be certain to list the FAX number and E-mail of the corresponding author on the title page. **All correspondence will be by E-mail and web site only.**

MANUSCRIPT should be typed in 12-point type and double spacing should be used throughout. It should carry an abstract of not more than 250 words including 4 paragraphs labeled: Background and aim of the work, Methods, Results, and Conclusions. Below the abstract provide 3-10 key words that will assist indexers in cross-indexing the article. Paragraphs to be set in a smaller type should be marked with an "s" (small) in the left hand margin. Avoid footnotes; when essential they are numbered consecutively and typed at the foot of the appropriate page.

ILLUSTRATIONS. It is the authors' responsibility to obtain permission (from the author and copyright holder) to reproduce illustrations, tables, etc. from other publications. Photographs and graphics should be sent as high resolution files: not less than 300 d.p.i. and with a base of the same size as a column of the Journal (8 cm). A letter of permission must accompany all photographs when there is a possibility of identification. Authors will pay for colour illustrations. Present rate for a full page colour illustration is about $ 600-1200. Final quotation will be given by the publisher. Legends should be typed on the "word" file of the manuscripts.

TABLES should be numbered consecutively with Arabic numerals. Type each table on a separate document, together with a brief caption. We do not welcome large tables of unanalysed data.

REFERENCES should be numbered consecutively in the order in which they appear in the text. 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. The list of references should be typed in numerical order and indicate: authors' names (all authors when six or less; when seven or more list only the first three and add "et al."); article title, name of the Journal (abbreviated as in Index Medicus), publication year, volume and first and last page numbers. Example:
Rizzato G, Marazzini L. Thoracoabdominal mechanics in elderly men. J Appl Physiol 1970; 28: 457-60.
If the reference is concerning a book, give authors' names, full title, name and address of publisher and publication year. Personal communications should not be included in the references, but may be cited in the text in parentheses.

COPYRIGHT. Please include a signed release of copyright to European Journal of Oncology with your text. Include the title of the article being submitted, as well as the date. Include the signature of coauthors.
The corresponding author must certify that the submitted manuscript is an original article and that he is able to prove this originality if required from the Referees. Without this declaration the manuscript will not be considered.

GALLEY PROOF. Unless indicated otherwise, galley proofs are sent to the first author and should be returned without delay. Alterations to galley proofs, other than those due to printer's error, are charged to the author. Accepted and rejected manuscripts are retained for six months after publication or rejection, then destroyed.

REPRINTS. Reprints are available at cost if they are ordered when the proof is returned. Order form and a price list are sent at request of the Author; payment must be made with the order.

NOTICE TO SUBSCRIBERS
European Journal of Oncology is published quarterly. Individual annual subscription for 2017 is 45,00 Euro in Italy, 55,00 Euro outside Italy. Institutional subscription is 50,00 Euro in Italy, 65,00 Euro outside Italy. The publisher accepts no responsibility for replacing Journal issues unless notified of non-receipt within 5 months of issue date. Payment should be made to the publisher: Mattioli 1885 srl, Strada di Lodesana 649/sx, Loc. Vaio, 43036 Fidenza (PR), Italy, Tel. 0039-(0)524-530383, Fax 0039-(0)524-82537, E-mail: subscribe@mattioli1885.com

COPYRIGHT
© European Journal of Oncology. All rights reserved. Accepted papers become the permanent property of European Journal of Oncology and no part may be reproduced, stored in a retrieval system or transmitted in any form or by any means without the prior permission of both the author and the publisher.

Autorizzazione del Tribunale di Parma n° 14/97 del 11/6/1997
ISSN 1128-6598

La banca dati viene conservata presso l'editore, che ne è titolare. La rivista viene spedita in abbonamento; l'indirizzo in nostro possesso verrà utilizzato per l'invio di questa o di altre pubblicazioni scientifiche. Ai sensi dell'articolo 10, legge 675/96, è nel diritto del ricevente richiedere la cessazione dell'invio e/o l'aggiornamento dei dati in nostro possesso.

La testata fruisce dei Contributi Statali diretti di cui alla legge 7 agosto 1990, n. 250

Mattioli 1885
srl- Strada di Lodesana 649/sx
43036 Fidenza (Parma)
tel 0524/530383
fax 0524/82537
www.mattioli1885.com

Direttore Generale
Paolo Cioni
Direttore Scientifico
Federico Cioni

Direttore Commerciale
Marco Spina
Formazione/ECM
Simone Agnello
Project Manager
Natalie Cerioli
Massimo Radaelli
Editing Manager
Anna Scotti
Editing
Valeria Ceci
Foreign Rights
Nausicaa Cerioli
Distribuzione
Massimiliano Franzoni

Journal Director /
Direttore Responsabile
Federico Cioni

Autorizzazione del Tribunale di Parma n. 14/97 del 11/6/1997
ISSN 1128-6598
La testata fruisce dei Contributi Statali diretti di cui alla legge 7 agosto 1990, n. 250

Contents

Volume 23 / n. 2 June 2018

Research

Case reports

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 65-71, 2018 © Mattioli 1885

Neutrophil to lymphocyte ratio and muscular invasion in early-stage bladder cancer: a meta-analysis

Massimo Madonia[1], Panagiotis Paliogiannis[1]*, Tatiana Solinas[1], Arduino Aleksander Mangoni[2], Ciriaco Carru[3], Angelo Zinellu[3]*

[1]Department of Medical, Surgical and Experimental Sciences, University of Sassari, Sassari, Italy; [2]Department of Clinical Pharmacology, College of Medicine and Public Health, Flinders University, Adelaide, Australia; [3]Department of Biomedical Sciences, University of Sassari, Sassari, Italy

Summary. *Background:* Bladder cancer is the ninth most common cancer worldwide. In its early stages, invasion of the muscle layer of the bladder is the major determinant for cystectomy. The aim of this meta-analysis was to evaluate the potential role of the neutrophil to lymphocyte ratio (NLR) in predicting muscular invasion in early-stage bladder cancer. *Methods:* A systematic literature search was conducted in Medline, Pubmed, Scopus and Clinicaltrials.gov databases for English language articles published in the last decade. *Results:* Five studies fulfilling the eligibility criteria were identified (1,612 participants, 1,217 with NMIBC and 395 with MIBC). Pooled results showed that NLR values were significantly higher in patients with MIBC (SMD: 0.45, 95% CI: 0.18-0.73; p=0.001). *Conclusions:* NLR is significantly higher in patients with MIBC in comparison to those with NMIBC. This simple, widely available and relatively inexpensive parameter might be useful for risk stratification in patients with early-stage BC.

Key words: cancer, bladder, urothelial, muscle invasion, NLR

Introduction

Bladder cancer (BC) is the ninth most common cancer worldwide, with approximately 430,000 new cases estimated in 2012 (1). Its incidence and prevalence peak in the seventh to eighth decade of life, and it is 3 to 4 times more common in men than in women. Incidence rates are highest in Europe, the United States, and Egypt (2). In the United States, the incidence and mortality rates have been stable during the past three decades. In 2012, an excess of 165,000 BC-related deaths were estimated worldwide (1). Urothelial cancer is the most common cancer of the bladder, accounting for more than 95% of bladder cancers in several populations (3, 4).

From a clinical perspective, BC is classified as non–muscle-invasive bladder cancer (NMIBC, 70%) and muscle-invasive bladder cancer (MIBC, 30%), because invasion of the muscle layer is the major determinant for performing a cystectomy (5). The pathological confirmation of muscular invasion derives from biopsies obtained using a transurethral approach (TUR). However, this approach has some limitations, especially in cases of repeated resections (6,7). For this reason, the identification of biomarkers associated with muscular invasion in early-stage BC might be particularly useful for risk stratification and management.

In recent years, the neutrophil to lymphocyte ratio (NLR), a simple, easy to perform, and relatively inexpensive parameter has attracted particular interest, because it accurately reflects systemic inflammatory alterations in numerous diseases, including cancer (8-12). Several authors have investigated the prognostic role of the NLR in bladder cancer (13-15). A number

* Contributed equally

of studies have also been performed to investigate differences in NLR values between BC patients with and without muscular invasion. We conducted a systematic review and meta-analysis of the studies investigating the associations between the NLR and muscular invasion in early-stage BC.

Materials and Methods

Eligibility criteria

Human studies were considered eligible if they met the following criteria: (1) pre-treatment assessment of blood NLR, (2) compared subjects with NIMBC and IMBC, (3) BC diagnosed histologically in accordance with the WHO classification system (16) and staged in accordance with the American Joint Committee on Cancer (AJCC) staging system (17), (4) English language, (5) full-text publications, (6) basic demographic and clinical data available.

Search strategy and study selection

A systematic literature search was conducted in Medline, Pubmed, Scopus and Clinicaltrials.gov databases for English language articles published between 2007 and 2017, using the following terms: "neutrophil to lymphocyte ratio" OR "NLR" AND "bladder cancer" OR "urothelial cancer".

Abstracts were independently screened by two investigators. If relevant, full articles were retrieved. References in these articles, citing relevant reviews or original studies were also accessed to identify additional eligible studies. Any disagreement between the reviewers was resolved by a third investigator. We used the Newcastle-Ottawa Scale (NOS) to assess the quality of each study (19). The Newcastle-Ottawa scale evaluated three components: selection of the cohort, comparability of cohorts on the basis of the design or analysis, and assessment of exposure and outcomes of interest. Studies achieving six or more stars were considered to be of high quality.

Statistical analysis

Standardized mean differences (SMD), and 95% confidence intervals (CIs), were used to construct forest plots of continuous data and to evaluate differences in NLR values between patients with NIMBC and patients with IMBC. $P<0.05$ was considered statistically significant. In one study (19) the standard deviation (SD) was estimated from the median and range.

Heterogeneity of SMD across studies was tested by using the Q statistic (significance level at $p<0.10$). The I^2 statistic, a quantitative measure of inconsistency across studies, was also calculated ($I^2<25\%$, no heterogeneity; I^2 between 25% and 50%, moderate heterogeneity; I^2 between 50% and 75%, large heterogeneity; and $I^2>75\%$, extreme heterogeneity) (20, 21). Due to the high heterogeneity, a random-effects model was used to calculate the pooled SMD and corresponding 95% confidence intervals.

To evaluate the presence of potential publication bias, the association between study size and magnitude of effect were analysed by means of Begg's adjusted rank correlation test and Egger's regression asymmetry test at the $p<0.05$ level of significance (22). Sensitivity analysis was conducted to investigate the influence of an individual study on the overall risk estimate by sequentially excluding one study in each step (23). Statistical analyses were performed using MedCalc for Windows, version 15.4 64 bit (MedCalc Software, Ostend, Belgium) and Stata 14 (STATA Corp., College Station, TX, USA). Reporting methods comply with the PRISMA statement.

Results

A flow chart describing the study selection is presented in Figure 1. We initially retrieved 281 studies. Of these, 132 duplicates were excluded after an initial screening, and 126 further studies were subsequently excluded after abstract evaluation. After full-text review of the remaining 23 articles, 18 studies were excluded because they did not meet the inclusion criteria. Five studies were included in the final meta-analysis (5, 19, 24-26).

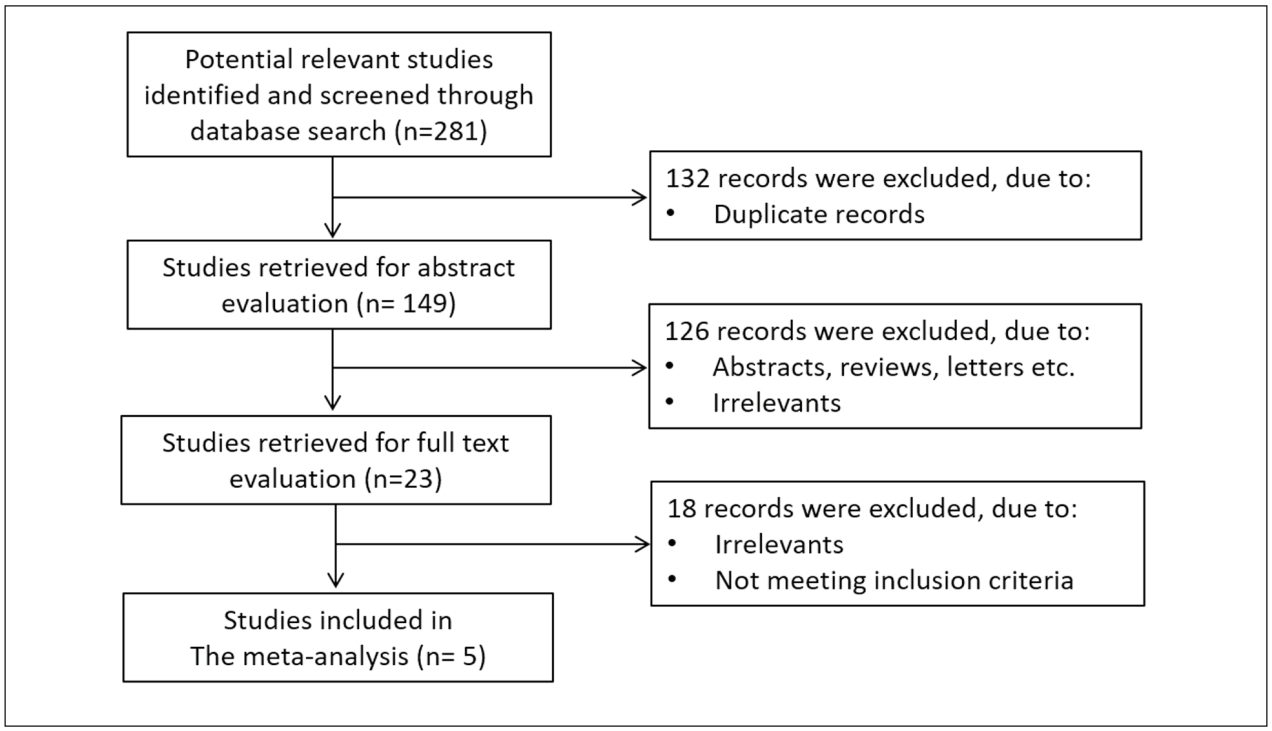

Figure 1. Flow chart showing the study search and selection procedure

Table 1. Summary of the studies on NMIBC vs MIBC included in the meta-analysis

First Author, Year, Country	Ceylan et al. 2014, Turkey	Kaynar et al. 2014, Turkey	Celik et al. 2016, Turkey	Ma et al. 2016, China	Tazeh et al. 2017, USA
Study design	R	R	R	R	R
NOS					
Patients enrolled (total n)	198	291	222	669	232
NMIBC					
n	162	192	162	579	122 (Ta)
Age (mean ±SD, years)	63±11.1	64±13.0	71.8± 10.9	65.2±11.9	NA
Gender (M/F)	150/12	156/36	141/21	NA	NA
NLR Mean±SD	3.36±2.9	2.4±0.1	3.44±2.0	2.71±2.5	2.2±1.0
MIBC					
n	36	99	60	90	110 (T2)
Age (mean ±SD, years)	72.6±10.3	75±10.0	75.7 ± 10.2	67±11.4	NA
Gender (M/F)	32/4	85/14	51/9	NA	NA
NLR Mean±SD	4.14±2.8	2.9±0.2	4.65±2.8	4.66±8.0	3.4±1.8

R: retrrospective; NA: not available

The characteristics of the five studies investigating the NLR in NMIBC vs MIBC, and their NOS evaluation, are presented in Table 1. All studies were retrospective and comprised a total of 1,612 patients, 1,217 with NMIBC and 395 with MIBC. The male/female ratio in the NMIBC and MIBC groups was not available in all studies.

The forest plot showed higher mean NLR values in MIBC patients, when compared to those with NMIBC, in all the studies (Figure 2). Because of the substantial heterogeneity between studies (I^2=80.1%, p <0.001), random-effects models were used. Pooled results showed that NLR values were significantly higher in patients with MIBC (SMD: 0.45, 95% CI: 0.18-0.73; p=0.001). No evidence of publication bias was noted (Begg, p=0.81; Egger, p=0.98). Results stability was evaluated through sensitivity analysis (Figure 3). The corresponding pooled SMD values were not substantially altered when single studies were removed, suggesting that the results of the meta-analysis were stable.

Discussion

Although the presence of leukocytes in tumour tissues was known since the 19th century, the evidence that inflammation plays a critical role in the genesis and progression of human cancers has only been generated during the last decade (27). The NLR is a simple index obtained from complete blood counts, which reflects the systemic inflammatory status of the organism, on the basis of disease-related modifications of the most representative cell populations of inflammation. It has been recently proposed as a predictor of the onset, progression, and prognosis of several chronic inflammatory diseases and cancers (8-15). Wei et al. performed a meta-analysis to investigate the prognostic role of NLR in urinary cancers, including renal cell, upper tract urothelial, prostate and bladder cancers (13). The authors found that elevated NLR was a poor predictor for survival in patients with urinary cancers. Similar results were reported in other meta-analyses

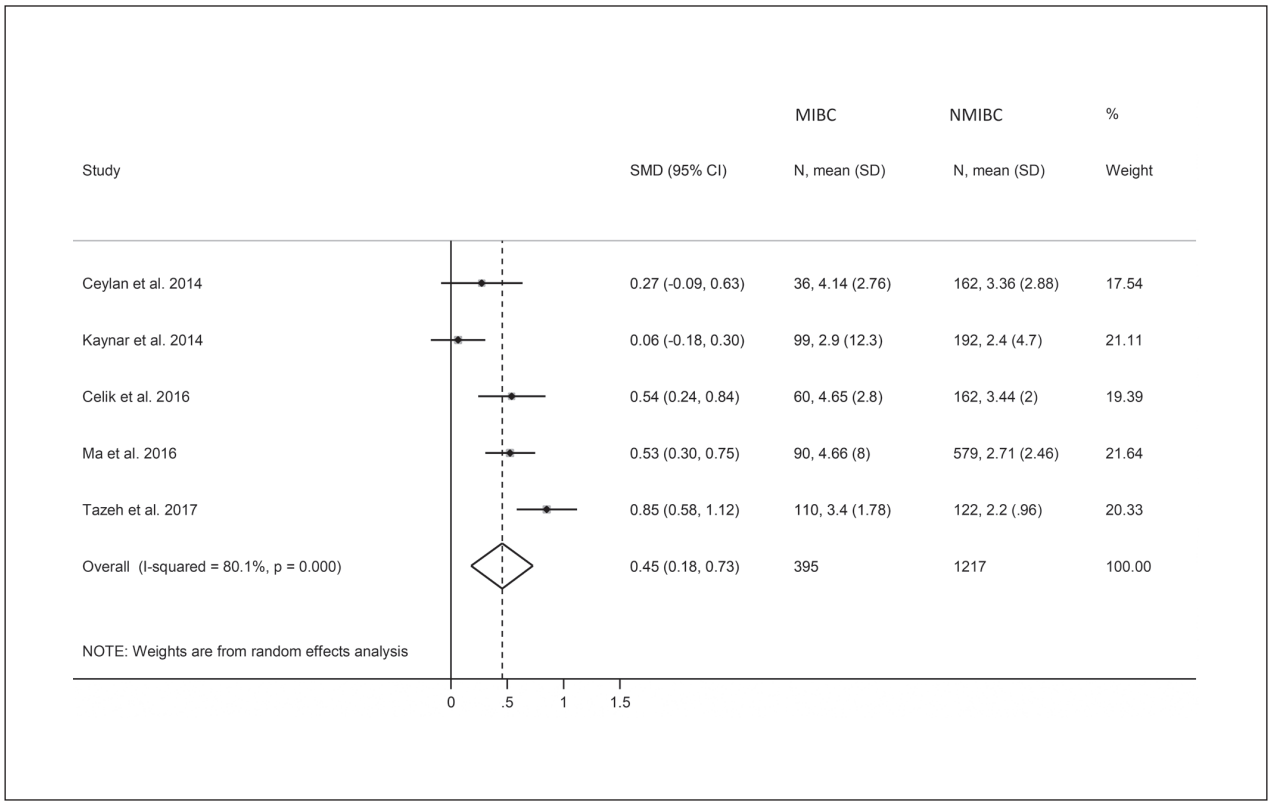

Figure 2. Forest plot of studies examining NLR and muscular invasiveness of early bladder cancer

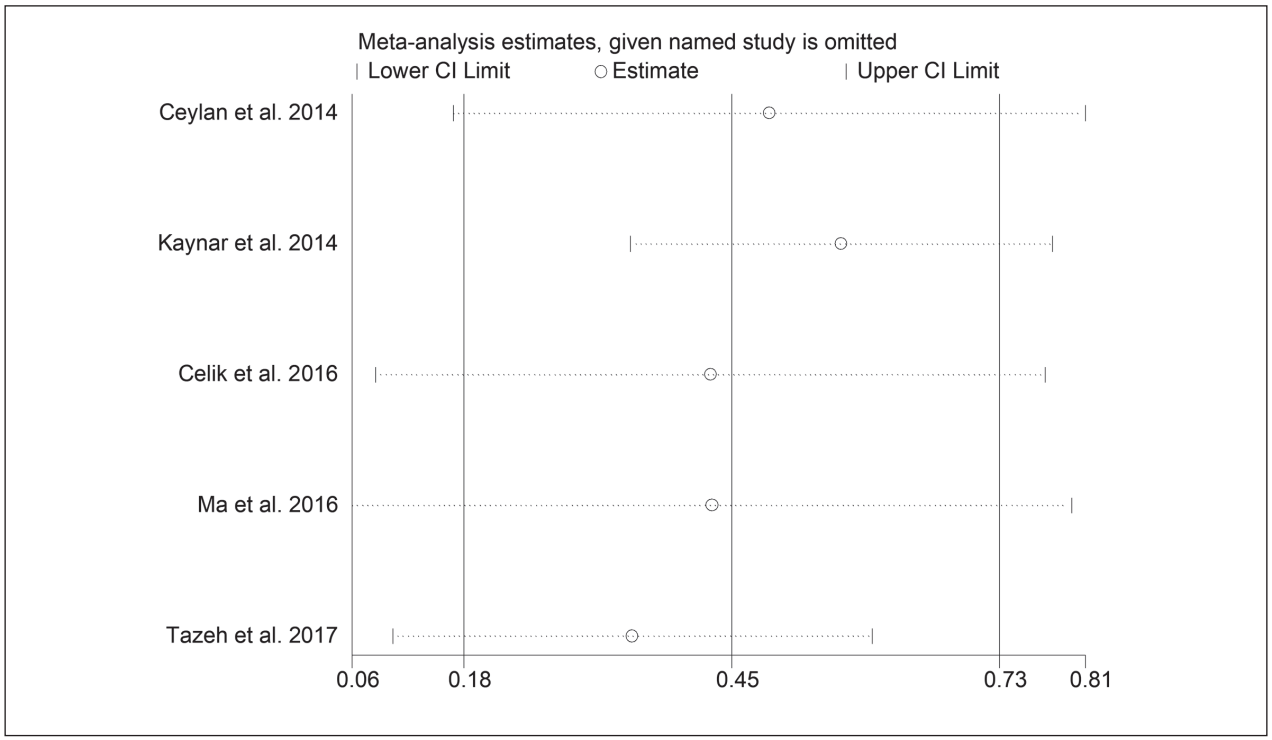

Figure 3. Sensitivity analysis of the association between NLR and muscular invasiveness of early bladder cancer

which analysed the prognostic roles of NLR in upper tract and urothelial BC (28) or urothelial BC alone (14, 29). These findings confirmed the potential prognostic roles of NLR in bladder cancer.

In this meta-analysis, we focused on a different potential clinical use of the NLR in early-stage BC, by comparing values between patients with and without muscular invasion. In five retrospective studies with adequate clinical, pathological and laboratory data, the NLR values were significantly higher in patients with MIBC (SMD: 0.45, 95% CI: 0.18-0.73; p=0.001) in comparison to those with NMIBC. This is likely to reflect the greater systemic inflammatory state in patients with muscular invasion, and thus, higher stage BC. Numerous previous studies have demonstrated the positive correlation between NLR levels and higher neoplastic stage in urinary and other malignancies (28, 30-32).

No consistent biases were detected by means of the Begg and Egger tests, however this may be influenced by the relatively low number of articles identified. Moreover, other limitations should be acknowledged, including the small number of studies identified and their retrospective design, which furthermore preclude meta-regression analyses. On the other hand, this is the first meta-analysis that evidences a potential role of NLR as a predictor of muscular invasion in early bladder cancer. The availability of such a predictor may be useful in clinical practice for the assessment of muscular invasion, especially in cases in which the pathological examination may be inconclusive. An additional advantage is the fact that NLR is a simple, low-cost, widely available index, which can be easily calculated from complete blood count tests. Nevertheless, further well-designed clinical trials are warranted to establish its potential clinical usefulness and applications.

Conclusions

The present meta-analysis, the first to evaluate the potential role of NLR in detecting muscular invasion of early stage BCs, showed that the NLR is consistently higher in patients with MIBC in com-

parison to those with NMIBC. Therefore, the NLR, a simple, widely available, and relatively inexpensive marker, may be useful in risk stratification and staging of patients with early-stage BC, especially those with a challenging pathological confirmation. Further prospective studies are required to confirm these findings.

References

1. http://globocan.iarc.fr. [accessed 12 November 2017].
2. Bray F, Ren JS, Masuyer E, Ferlay J. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int J Cancer 2013; 132: 1133-45.
3. Malats N, Real FX. Epidemiology of bladder cancer. Hematol Oncol Clin North Am 2015; 29: 177-89.
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
5. Ceylan C, Doluoglu OG, Keleş I, et al. Importance of the neutrophil-to-lymphocyte ratio in muscle-invasive and non-muscle invasive bladder tumors. Urologia 2014; 81: 120-4.
6. Jakse G, Algaba F, Malmström PU, Oosterlinck W. A second- look TUR in T1 transitional cell carcinoma: why? Eur Urol 2004; 45: 539-46.
7. Brauers A, Buettner R, Jakse G. Second resection and prognosis of primary high risk superficial bladder cancer: is cystectomy often too early? J Urol 2001; 165: 808-10.
8. Paliogiannis P, Fois AG, Sotgia S, et al. Neutrophil to lymphocyte ratio and clinical outcomes in COPD: recent evidence and future perspectives. Eur Respir Rev 2018; 27: 170113.
9. Mertoglu C, Gunay M. Neutrophil-Lymphocyte ratio and Platelet-Lymphocyte ratio as useful predictive markers of prediabetes and diabetes mellitus. Diabetes Metab Syndr 2017; 11: S127-S131.
10. Paliogiannis P, Scognamillo F, Bellomo M, et al. Neutrophil to lymphocyte ratio as a predictor of thyroid papillary carcinoma. Acta Med Mediterr 2015; 31: 371-5
11. Dolan RD, Lim J, McSorley ST, Horgan PG, McMillan DC. The role of the systemic inflammatory response in predicting outcomes in patients with operable cancer: Systematic review and meta-analysis. Sci Rep 2017; 7: 16717.
12. Dolan RD, McSorley ST, Horgan PG, Laird B, McMillan DC. The role of the systemic inflammatory response in predicting outcomes in patients with advanced inoperable cancer: Systematic review and meta-analysis. Crit Rev Oncol Hematol 2017; 116: 134-46.
13. Wei Y, Jiang YZ, Qian WH. Prognostic role of NLR in urinary cancers: a meta-analysis. PLoS One 2014; 9: e92079.
14. Lucca I, Jichlinski P, Shariat SF, et al. The Neutrophil-to-lymphocyte Ratio as a Prognostic Factor for Patients with Urothelial Carcinoma of the Bladder Following Radical Cystectomy: Validation and Meta-analysis. Eur Urol Focus 2016; 2: 79-85.
15. Buisan O, Orsola A, Oliveira M, et al. Role of Inflammation in the Perioperative Management of Urothelial Bladder Cancer With Squamous-Cell Features: Impact of Neutrophil-to-Lymphocyte Ratio on Outcomes and Response to Neoadjuvant Chemotherapy. Clin Genitourin Cancer 2017; 15: e697-e706.
16. Sauter G, Algaba F, Amin M, et al. Tumours of the urinary system: non-invasive urothelial neoplasias. In: Eble JN, Sauter G, Epstein JI, Sesterhenn IA, editors. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. 1st ed. Lyon, France: IARC Press, 2004. p. 110-23.
17. Members of the TNM Prognostic Factors Core Group. Urological tumours: urinary bladder. In: Sobin LH, Gospodarowicz M, Wittekind C, editors. UICC International Union Against Cancer TNM classification of malignant tumors. 7th ed. Oxford, UK: Wiley-Blackwell, 2009. p. 262-5.
18. Wells G, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. 2013. http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.
19. Kaynar M, Yıldırım ME, Badem H, et al. Bladder cancer invasion predictability based on preoperative neutrophil-lymphocyte ratio. Tumour Biol. 2014; 35: 6601-5.
20. Bowden J, Tierney JF, Copas AJ, Burdett S. Quantifying, displaying and accounting for heterogeneity in the meta-analysis of RCTs using standard and generalised Q statistics. BMC Med Res Methodol 2011; 11: 41.
21. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002; 21: 1539–58
22. Tobias A. Assessing the influence of a single study in the meta-analysis estimate. Stata Technical Bulletin 1999; 47: 15–17.
23. Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. Biometrics 1994; 50: 1088-101.
24. Celik O, Akand M, Keskin MZ, Yoldas M, Ilbey YO. Preoperative neutrophil-to-lymphocyte ratio (NLR) may be predictive of pathologic stage in patients with bladder cancer larger than 3 cm. Eur Rev Med Pharmacol Sci 2016; 20: 652-6.
25. Ma C, Lu B, Diao C, Zhao K, Wang X, Ma B, Lu B, Sun E. Preoperative neutrophil-lymphocyte ratio and fibrinogen level in patients distinguish between muscle-invasive bladder cancer and non-muscle-invasive bladder cancer. Onco Targets Ther 2016; 9: 4917-22.
26. Tazeh NN, Canter DJ, Damodaran S, et al. Neutrophil to Lymphocyte Ratio (NLR) at the Time of Transurethral Resection of Bladder Tumor: A Large Retrospective Study and Analysis of Racial Differences. Bladder Cancer 2017; 3: 89-94.
27. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010; 140: 883-99.

28. Li X, Ma X, Tang L, et al. Prognostic value of neutrophil-to-lymphocyte ratio in urothelial carcinoma of the upper urinary tract and bladder: a systematic review and meta-analysis. Oncotarget 2016; 8: 62681-92.
29. Tang X, Du P, Yang Y. The clinical use of neutrophil-to-lymphocyte ratio in bladder cancer patients: a systematic review and meta-analysis. Int J Clin Oncol 2017; 22: 817-25.
30. Wang L, Liang D, Xu X, et al. The prognostic value of neutrophil to lymphocyte and platelet to lymphocyte ratios for patients with lung cancer. Oncol Lett 2017; 14: 6449-56.
31. Margetts J, Ogle LF, Chan SL, et al. Neutrophils: driving progression and poor prognosis in hepatocellular carcinoma? Br J Cancer 2018;118: 248-57.
32. Sahin AG, Aydin C, Unver M, Pehlivanoglu K. Predictive Value of Preoperative Neutrophil Lymphocyte Ratio in Determining the Stage of Gastric Tumor. Med Sci Monit 2017; 23: 1973-9.

Correspondence:
Dr. Panagiotis Paliogiannis, MD, PhD.
Department of Medical, Surgical and Experimental Sciences
University of Sassari,
V.le San Pietro 43b - 07100 Sassari, Italy
Tel. +393405931590
Fax +30079228503
E-mail: panospaliogiannis@gmail.com

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 72-79, 2018 © Mattioli 1885

Elotuzumab in multiple myeloma: a single centre experience

Monica Galli[1], Paola Stefanoni[1], Laura Paris[1], Chiara Pavoni[1], Federica Delaini[1], Alessandro Rambaldi[1, 2]

[1]Hematology & Bone Marrow Transplant Unit, ASST Papa Giovanni XXIII, Bergamo, Italy; [2]Department of Oncology & Hematology, Università Statale di Milano, Milan, Italy

Summary. *Background and aims of the work:* Elotuzumab is a first-in-class immunostimulatory monoclonal antibody approved in Italy in April 2017 for use in combination with lenalidomide and dexamethasone (ELd) for relapsed/refractory multiple myeloma (MM). We present our single Centre experience with ELd in patients with MM, focusing on the determinants driving the choice of the most appropriate second-line treatment. *Methods:* We performed a retrospective analysis of patients experiencing a first relapse in the Hematology and Bone Marrow Transplant Unit of the Papa Giovanni XXIII hospital in Bergamo, Italy between April and December of 2017. *Results:* We tended to administer ELd treatment to young and fit patients with non-aggressive relapsed/refractory MM. In general, ELd was well tolerated. We present details of 2 illustrative cases. *Conclusions:* The immunostimulatory effects and favorable clinical toxicity profile of elotuzumab make it an ideal drug against MM. Ongoing clinical trials will elucidate its most appropriate placement and its best combination partners to improve disease control and, therefore, the duration and quality of life.

Key words: multiple myeloma, treatment, elotuzumab

Introduction

Multiple myeloma (MM) is a largely incurable tumor (1) resulting from the proliferation of monoclonal plasma cells in the bone marrow. Its incidence rate is about 8 cases per 100,000 people in Italy (2) and it has an estimated mortality rate of 2.2 cases per 100,000 people in Europe (3). Significant improvement in survival has been obtained (4), mostly due to the incorporation of autologous stem cell transplantation (ASCT) in the 1980s (5) and the availability of an increasing number of novel agents starting from 1990s. These agents, which include immunomodulatory drugs, proteasome inhibitors, monoclonal antibodies, histone deacetylase inhibitors, kinase inhibitors, heat shock protein inhibitors, signal transduction pathway inhibitors, have been used alone or in various combinations both in newly diagnosed and in relapsed/refractory MM (reviewed in 6).

Elotuzumab is a fully humanized IgGκ monoclonal antibody directed toward the extracellular region of CS1, which is a cell surface glycoprotein receptor member of the signaling lymphocytic activation molecule (SLAM) family (7). CS1 is expressed on natural killer (NK) cells, B- and T-lymphocytes, dendritic cells and monocytes, and is overexpressed in MM plasma cells (7). CS1 engagement by elotuzumab on MM plasma cells and NK cells leads to antibody-dependent cellular cytotoxicity and direct NK cell activation. CS1 has a critical role in the phagocytosis of hematopoietic tumor cells by macrophage (7), which implies another possible mechanism of action for elotuzumab. Finally, binding of elotuzumab to monocytes inhibits the production of proinflammatory cytokines (8).

Pre-clinical studies showed elotuzumab to be effective both *in vitro* against MM cells and *in vivo* in animal models of MM (9, 10). This evidence prompted clinical investigation of elotuzumab in MM patients.

Ineffective when used alone (11), elotuzumab combined with bortezomib and dexamethasone showed limited efficacy in pre-treated MM patients (12). In contrast, the randomized ELOQUENT-2 clinical trial showed that lenalidomide and dexamethasone are efficient partners of elotuzumab in relapsed/refractory MM (13, 14). Updates of the ELOQUENT-2 study in 2017 (15) and 2018 (16) confirmed that the combination of elotuzumab, lenalidomide and dexamethasone (ELd) significantly improved all clinical outcomes when compared to lenalidomide and dexamethasone alone (Ld). Finally, serum M-protein dynamic modeling predicted less tumor regrowth with ELd. Adverse events were comparable between the 2 arms, indicating that elotuzumab did not cause additional toxicity. In 2015, these data led to the approval of ELd for treatment of MM in patients with 1-3 prior therapies in the United States, and patients with ≥1 prior line of treatment in Europe. In April of 2017, the Italian Medicines Agency (AIFA) approved ELd in patients with MM who had received at least one prior therapy.

We present our single Centre experience with ELd in patients with relapsed/refractory MM. In particular, we discuss the determinants of choice of ELd as second-line treatment, its tolerability profile and the major outcomes of patients receiving ELd irrespective of their previous treatment. Two clinical cases are also presented and discussed.

Determinants of choice of ELd as second-line treatment of relapsed/refractory MM patients

Presently in Italy, the following treatments are available in ≥ second-line: bortezomib and dexamethasone (Bd); Ld either alone or in combination with either carfilzomib (KLd) or elotuzumab (ELd); daratumumab and dexamethasone in combination with either lenalidomide or bortezomib. Although algorithms have been proposed to facilitate treatment decisions (17), the choice may still be difficult in individual patients. Indeed, both patient-related and disease-related factors must be considered to identify the treatment with the best risk-benefit ratio.

We evaluated the determinants of second-line treatment choice in a retrospective analysis of patients experiencing a first symptomatic MM progression/relapse in the Hematology and Bone Marrow Transplant Unit of the Papa Giovanni XXIII hospital in Bergamo, Italy between April and December of 2017. At that time, daratumumab combinations were not available according to AIFA regulations. Demographic and disease characteristics at MM diagnosis of the 31 patients are reported in Table 1; their first-line treatments are detailed in Table 2.

Table 1. Demographic and disease characteristics at MM diagnosis.

Characteristic	
Gender, M / F	16 / 15
Age, yrs median (range)	70 (48 - 85)
Isotype, n	
A	6
D	2
G	17
LC	6
Light chain, n	
κ / λ	19 / 12
Durie & Salmon stage, n	
I	1
II	5
III	25
A / B	24 / 7
International Staging System, n	
1	13
2	6
3	10
na	2

Table 2. First-line treatments of 31 patients with MM.

Treatment	Pts, n
High-Dose Therapy + Autotransplant	13
VTD	10
Ld	1
KCyD	1
VAD	1
Non-Autotransplant programs	18
VMP	14
TMP	2
Ld	1
KLd	1

Median progression free survival (PFS) on first-line treatment had been 18.4 months (range 1.7-150.2 months), and the median time to next treatment (TTNT) was 22.6 months (range 2.0-271.8 months). The second-line treatments are shown in Figure 1. One patient died a few days after relapse and was excluded from analysis. Clinical and biological characteristics of the remaining 30 patients were grouped according to the type of second-line treatment (Table 3). On average, patients receiving a 2-drug combination were 10 years older and had higher frailty scores (18) (median 2 vs 1) than patients receiving a 3-drug combination. Among patients treated with a 3 drug combination, those in the ELd group had longer PFS and TTNT compared to those treated with KLd. Patients in this latter group had lower values of hemoglobin and platelets and higher LDH values. The only patient with a plasma cell leukemia relapse had received KLd. This patient did not respond to treatment and died soon after the second KLd course. At last follow-up, 21 patients (70%) were still on treatment. Overall, 9 out of 11 patients (82%) treated with ELd reached a > partial remission, and 6 patients are still on treatment. Two patients in good partial remission stopped ELd treatment after 5 and 6 courses, respectively, to undergo a second high-dose therapy with ASCT procedure. The main post-transplant complication was reactivation of cytomegalovirus, which occurred in one patient and was well-controlled with specific antiviral therapy. Thus, ELd appears to be a feasible second-line treatment for controlling MM, and may be a safe bridge to ASCT.

In conclusion, we choose a 2-drug combination (Ld or Bd) for elderly and frail patients, whereas a 3-drug combination (ELd or KLd) was reserved for younger and fitter patients. However, more than one year of experience with ELd has increased our confidence in its tolerability profile (see below), and is prompting us to consider ELd also for second-line treatment of elderly and/or frail patients. Patient preference for a completely oral regimen had a major influence on the choice of Ld over ELd. The choice between 3-drug combinations was mostly determined by the biology of each MM relapse, with ELd used in less aggressive cases. However, ELd was effective also in aggressive relapse of MM (see Clinical Case 2 below).

Outcome of MM patients treated with ELd

The ELOQUENT-2 clinical trial (14-16) showed that ELd treatment improved the overall response rate (79% vs 66%, p=0.0002) and reduced the risk of pro-

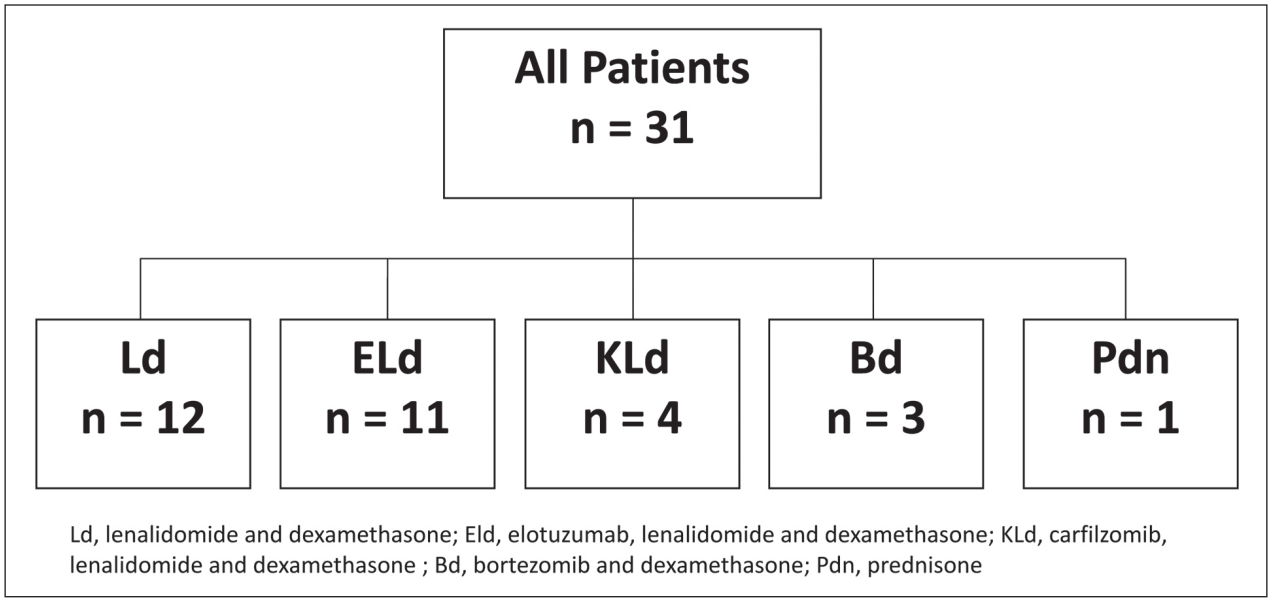

Figure 1. Second-line treatments received by the 31 MM patients.

Table 3. Patient characteristics at first symptomatic relapse according to second-line treatment selected (n = 30).

Characteristic	Ld N = 12	ELd N = 11	KLd N = 4	Bd N = 3
Gender, M / F	7 / 5	4 / 7	3 / 1	1 / 2
Age, years	77 (48-85)	68 (57-78)	64 (53-80)	77 (67-78)
PFS, months	12.3 (1.7-37.3)	24.9 (14.4-150.2)	18.5 (11.6-91.8)	44.0 (15.7-64.7)
TTNT, months	15.0 (2.0-38.2)	37.0 (15.3-271.8)	19.8 (11.8-99.1)	44.0 (16.5-79.6)
ECOG	1 (0-2)	0 (0-2)	0 (0-2)	2 (1-2)
CIRS	3 (0-5)	2 (0-4)	1 (0-7)	2 (2-2)
Frailty score	2 (0-4)	1 (0-3)	1 (0-2)	2 (1-3)
Hemoglobin, g/dl	11.8 (8.1-14.7)	12.1 (8.0-15.0)	10.9 (7.8-14.8)	10.2 (9.5-10.9)
WBC, x 10^3/ml	6.4 (2.6-15)	7.6 (4.3-17.3)	6.7 (4.0-38.5)	7 (5.6-8.4)
Neutrophil count, x 10^3/ml	4.4 (1.1-11.2)	5.8 (2.1-15.5)	3.3 (2.4-3.4)	4.5 (4.0-4.9)
Platelet count, x 10^3/ml	167 (71-307)	215 (8-291)	63 (6-254)	226 (91-253)
LDH value, U/l	451 (308-775)	393 (285-1062)	659 (344-4161)	381 (288-1229)
Serum creatinine, mg/dl	0.85 (0.62-4.05)	0.76 (0.54-2.32)	0.86 (0.84-1.74)	0.89 (0.65-0.99)
Bone marrow plasma cells, %	35 (2-80)	70 (5-90)	10 (5-60)	60 (40-80)
Cycles, n median (range)	9 (1-13)	6 (1-13)	2 (1-9)	8 (2-9)
Best outcome				
CR	1	1		1
VGPR	1	3	1	
GPR	2	4		1
PR	5	1		
SD	1	1	2	1
PD	2	1	1	
Death	1	1	2	1

PFS, progression-free survival; TTNT, time to next treatment; ECOG PS, Eastern Cooperative Oncology Group performance status; CIRS, Cumulative Illness Rating Scale; CR, complete remission; VGPR. Very good partial remission; GPR, good partial remission; PR, partial remission; SD, stable disease; PD, progressive disease

gression/death by 27% (Hazard Ratio 0.73; p=0.0014) compared to Ld. Furthermore, overall survival showed a significant trend in favor of ELd (p=0.0257), with 1-, 2-, 3- and 4-year rates of 91% vs 83%, 73% vs 69%, 60% vs 53% and 50% vs 43%, respectively.

Between April 2017 and June 2018 we have treated 18 patients with ELd. Details of 11 of them have already been discussed above. Nine of the 18 patients had one or more comorbid conditions, and their frailty score was 1 or 2 in 8 cases. Although AIFA had not set a limit on the number of previous treatments above which ELd cannot be prescribed, we chose to administer it early so that elotuzumab can fully elicit its immunostimulatory effects. Indeed, more than 70% of our patients (13/18) received ELd as second-line treatment. The others received it as third-line (3), fourth-line (1) and fifth-line (1). A median of 6 cycles (range, 1-14) were administered. During treatment, we documented 2 complete remissions, 6 very good partial remissions, 4 good partial remissions, 3 partial remissions, one stable disease and one progressive disease. The overall response rate (i.e., ≥ partial remission) was 83%. As of June 2018, 12 patients were still receiving ELd treatment. Two patients discontinued treatment, one after the first cycle for an adverse event and the other after the second cycle for MM progression (see "Tolerability of ELd" below). Two patients died: one after the first ELd cycle, due to worsening general health, despite a decrease of free κ light chains from 2843 to 8.3 mg/L; the other after the second cycle, due

to MM progression. Thus, 1-year overall survival was 89%. These favorable outcomes – obtained outside of a clinical trial – closely resemble those of the ELOQUENT-2 study (14-16).

Tolerability of ELd

The ELOQUENT-2 clinical trial randomly assigned 321 patients to receive ELd (14-16) and established the good safety and tolerability of the ELd combination. Infusion reactions occurred mainly during the first dose and affected 10% of the patients, with only 1% Grade 3 and no Grade 4-5 reactions. Two patients (1%) discontinued elotuzumab because of infusion reactions.

Our premedication scheme before elotuzumab infusion followed the main indications from the ELOQUENT-2 study: 1. oral dexamethasone on the day before elotuzumab; 2. intravenous combination of dexamethasone, ranitidine, chlorophenamine and paracetamole before the elotuzumab infusion; 3. the first elotuzumab infusion is administered with progressively increasing infusion rate. If no reaction occurred, subsequent infusions were completed in about 60 minutes. Using this scheme, we did not observe any Grade ≥2 infusion reactions among the 18 patients treated with ELd in our Centre. Once good control of MM has been obtained, we administer dexamethasone only before elotuzumab (i.e., every 15 days), to reduce the monthly dexamethasone dose and the risk of steroid-related side effects.

Virtually all patients enrolled in the ELOQUENT-2 clinical trial experienced adverse events. Fatigue and diarrhea were the most common non-hematological events, whereas lymphocytopenia, neutropenia, anemia, and thrombocytopenia were the main hematological events. The prevalence of Grade 3-4 hematological and non-hematological adverse events was similar in the two study arms. In particular, the exposure-adjusted incidence rates per 100 patient-years for infection were 198 and 192 for ELd and Ld, respectively; for second primary malignancies, these rates were 5 and 3, respectively. Herpes zoster infections were more common in patients treated with ELd. Anti-herpetic prophylaxis is recommended for all patients treated with ELd (19).

As of June 2018, we have administered 129 ELd cycles to 18 patients. In our experience, only one patient permanently stopped elotuzumab infusions, and this was due to an adverse psychiatric event during the first cycle. The patient was switched to Ld treatment, which was also not tolerated and had to be interrupted. Thus, we attributed these psychiatric symptoms to dexamethasone, rather than elotuzumab. Two patients developed pneumonia, and required a transient interruption of ELd treatment: one of them was receiving ELd as fourth-line treatment after allogeneic bone marrow transplantation. All of our patients received acyclovir and acetylsalicylic acid prophylaxis and, so far, none has experienced reactivation of latent herpes virus or thrombotic events. Two patients are receiving monthly immunoglobulin infusions because of recurrent upper respiratory tract infections and hypogammaglobulinemia. No primary secondary cancers have occurred.

First clinical case

This male patient was diagnosed with IgGκ monoclonal gammopathy of undetermined significance in 1998 at the age of 38 years. Progression to symptomatic MM, Durie and Salmon (D&S) stage I-A, International Staging System (ISS) 1, was documented in 2003. In December 2004 he remained in D&S stage I-A, but his serum monoclonal component (sMC) had increased to 4.23 g/dl, proteinuria was 2.59 g/d (Bence Jones 136 mg/dl) and bone marrow aspirate showed a 60% infiltration by plasma cells. Bone involvement was absent. After discussion, the patient accepted first-line treatment consisting of induction with 3 cycles of thalidomide and dexamethasone, mobilization with cyclophosphamide 7 g/m^2 followed by peripheral stem cell collection, and consolidation with double ASCT following melphalan 200 mg/m^2. At the end of this treatment (December 2005) a very good complete remission was documented, and regular follow-up was started without maintenance therapy. In August 2009 (+45 months after the second ASCT), progression was documented as reappearance of isolated sMC that was not measurable, but positive at immunofixation. The patient was followed without treatment: in November 2011 (+72 months) sMC was 1.08 g/dl; in April 2016

(+124 months) sMC had increased to 2 g/dl. His general health was excellent: no bone pain was reported; blood cell counts, serum calcium and renal function were all normal. This situation was maintained until April 2017 (+136 months), when sMC rose to 2.61 g/dl (details in Table 4). By that time, the patient was 57 years old, still in excellent health and naïve to both bortezomib and lenalidomide. Therefore, several options could be proposed for second-line treatment according to AIFA rules:

1. Bd cycles, alone or in combination with bendamustine;
2. Ld cycles, alone or in combination with carfilzomib or elotuzumab.

Young age and favorable performance status (PS) prompted us to exclude a 2-drug combination approach. Among the 3-drug combinations, the absence of high-risk cytogenetics and the extremely slow biochemical progression favored ELd. The patient is now receiving the seventeenth ELd cycle, with a stable good partial remission characterized by the persistence of an isolated sMC of about 0.5-0.6 g/dl.

Second clinical case

This female patient was diagnosed with IgDλ MM, D&S III-A stage, ISS 1, in 2015 at the age of 64 years. Her first-line treatment consisted of induction with 4 cycles of bortezomib – thalidomide – dexamethasone, mobilization with cyclophosphamide 2 g/m^2, peripheral blood stem cell collection (2.9x10^6 CD34+ cells/kg body weight), and consolidation with a single ASCT following melphalan 200 mg/m^2. No maintenance was given. Complete remission was obtained, which lasted 16 months. Features of MM relapse are detailed in Table 5. At the time of relapse, she was hospitalized for acute coronary syndrome complicated by severe anemia requiring transfusional support. Treatment started with intravenous dexamethasone 20 mg/d for 4 consecutive days. After discharge from the cardiology unit, she started treatment with ELd. The

Table 5. Clinical data from case 2 at first relapse, start of ELd therapy and last visit.

Laboratory data	Relapse	Last visit
Hemoglobin, g/L	80	125
Platelets/mm^3	8,000	262,000
Serum IgDλ MC, g/dl	2.02	Negative
Proteinuria, g/d	0.07	Negative
Bence Jones, g/l	Positive	Negative
Serum free λ light chain, mg/L	1,455	5.61
Bone marrow plasma cells, %	>90	nd*
LDH, U/L	927	Normal
Renal function & serum calcium	Normal	Normal
Hepatic function	Normal	Normal

*ND, not determined

Table 4. Clinical data from case 1 at first relapse, start of ELd therapy and last visit.

Laboratory data	Relapse	Start of ELd	Last visit
Hemoglobin, g/L	154	152	117
Platelets/mm^3	219,000	265,000	255,000
IgGk MC, g/dl	IF+	2.61	0.6
Proteinuria, g/d	0	0.57	0.2
Bence Jones, g/d	Negative	0.4	Negative
Bone marrow plasma cells, %	nd*	60	nd
FISH	Nd	1(q21)	nd
LDH, U/L	Nd	Normal	Normal
Serum creatinine, mg/dl	0.84	0.84	0.9
Hepatic function	Normal	Normal	Normal
Skeletal CT	Nd	Small, diffuse osteolytic lesions	Nd

*Not determined

choice of this treatment was based on the following issues:

1. Relapse was aggressive and occurred less than two years after the ASCT. This led us to exclude a 2-drug combination strategy;
2. Similarly, we did not consider bortezomib – dexamethasone – bendamustine because of her previous exposure to bortezomib;
3. Due to the acute coronary syndrome, a 3-drug lenalidomide and dexamethasone-based combination required the reduction of the starting dose of lenalidomide to 15 mg/d (with the usual schedule of 21 days on and 7 days off drug). We excluded the use of carfilzomib, due to its potential cardiotoxicity;
4. At the time of relapse, daratumumab was not licensed in Italy.

Hematological and general health conditions improved quickly, with thrombocytopenia normalizing after the first ELd cycle, and hemoglobin level rising above 12 g/dl after the third cycle. Complete remission was documented as the disappearance of the sMC and normalization of the serum free light chain ratio at the end of the fifth cycle; this has been maintained through the 18 cycles.

This case clearly shows that ELd can be effective for an aggressive MM relapse, suggesting that its use need not be confined to slowly progressing MM.

Future uses of elotuzumab in MM

In October 2016, AIFA approved Ld cycles for the first-line treatment of patients not eligible for ASCT; in June 2018, they approved lenalidomide monotherapy as maintenance in the post-ASCT setting. Thus, an increasing number of MM patients will receive lenalidomide early in the course of their disease, which implies major changes in the use of elotuzumab in the near future. Several possible scenarios can be envisioned:

1. ELd may also move to front-line treatment for MM. This is already being investigated in the phase III ELOQUENT-1 clinical trial, which is comparing ELd cycles vs Ld in newly diagnosed, non-ASCT eligible patients (20). A phase I study is currently investigating the effect of the combination of elotuzumab with bortezomib, lenalidomide and dexamethasone in newly diagnosed high-risk patients (21).
2. Elotuzumab may move to post-ASCT maintenance. In this setting a phase II study is currently investigating its effect in combination with lenalidomide (22).
3. Elotuzumab may move even further upfront and be used in smoldering MM. Indeed, clinical trials are ongoing with elotuzumab monotherapy (23) and with the ELd combination in high-risk smoldering MM (24).
4. Elotuzumab may change partners. The ELOQUENT-3 phase II clinical trial is currently recruiting patients with relapsed/refractory MM to compare elotuzumab, pomalidomide and dexamethasone with pomalidomide and dexamethasone alone. Another study is evaluating the combination of elotuzumab with thalidomide and dexamethasone in relapsed/refractory MM patients (25). Other monoclonal antibodies may be potentially interesting partners of elotuzumab. Studies combining elotuzumab with nivolumab (a PD-1 checkpoint inhibitor), lirilumab (directed against KIR2D), or urelumab (directed against CD137) are recruiting patients.

In conclusion, elotuzumab is a first-in-class monoclonal antibody approved for relapsed/refractory MM. Its CS1-mediated immunostimulatory effects and favorable toxicity profile make elotuzumab an ideal drug against MM. Ongoing clinical trials will elucidate its most appropriate placement and its ideal companions in order to improve disease control and, therefore the duration and quality of life for patients with MM.

References

1. Ravi P, Kumar SK, Cerhan JR, et al. Defining cure in multiple myeloma: a comparative study of outcomes of young individuals with myeloma and curable hematologic malignancies. Blood Cancer J 2018; 8: 26.
2. AIRTUM Working Group. Italian cancer figures – Report 2015: the burden of rare cancers in Italy. Epidemiol Prev

2018; 40 (1 Suppl 2): 1-120. [see errata corrige: Epidemiol Prev 2016;40:83].
3. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013; 49: 1374-403.
4. Costa LJ, Brill IK, Omel J, Godby K, Kumar SK, Brown EE. Recent trends in multiple myeloma incidence and survival by age, race, and ethnicity in the United States. Blood Adv 2017; 4: 282-87.
5. Raza S, Safyan RA, Rosenbaum E, Bowman AS, Lentzsch S. Optimizing current and emerging therapies in multiple myeloma: a guide for the hematologist. Ther Adv Hematol 2017; 8: 55-70.
6. Larocca A, Mina R, Gay F, Bringhen S, Boccadoro M. Emerging drugs and combinations to treat multiple myeloma. Oncotarget 2017; 8: 60656-72.
7. Chen J, Zhong MC, Guo H, et al. SLAMF7 is critical for phagocytosis of haematopoietic tumour cells via Mac-1 integrin. Nature 2017; 544: 493-97.
8. Malaer JD, Mathew PA. CS1 (SLAMF7, CD319) is an effective immunotherapeutic target for multiple myeloma. Am J Cancer Res 2017; 7: 1637-41.
9. Hsi ED, Steinle R, Balasa B, et al. CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. Clin Cancer Res 2008; 14: 2775-84.
10. van Rhee F, Szmania SM, Dillon M, et al. Combinatorial efficacy of anti-CS1 monoclonal antibody elotuzumab (HuLuc63) and bortezomib against multiple myeloma. Mol Cancer Ther 2009; 8: 2616-24.
11. Zonder JA, Mohrbacher AF, Singhal S, et al. A phase 1, multicenter, open-label, dose escalation study of elotuzumab in patients with advanced multiple myeloma. Blood 2012; 120: 552-59.
12. Jakubowiak AJ, Benson DM, Bensinger W, et al. Phase I trial of anti-CS1 monoclonal antibody elotuzumab in combination with bortezomib in the treatment of relapsed/refractory multiple myeloma. J Clin Oncol 2012; 30: 1960-65.
13. Lonial S, Vij R, Harousseau JL, et al. Elotuzumab in combination with lenalidomide and low dose dexamethasone in relapsed or refractory multiple myeloma. J Clin Oncol 2012; 30: 1953-9.
14. Lonial S, Dimopoulos M, Palumbo A, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. N Engl J Med 2015; 373: 621-31.
15. Dimopoulos MA, Lonial S, White D, et al. Elotuzumab plus lenalidomide/dexamethasone for relapsed or refractory multiple myeloma: ELOQUENT-2 follow-up and post-hoc analyses on progression-free survival and tumour growth. Br J Haematol 2017; 178: 896-905.
16. Dimopoulos MA, Lonial S, Betts KA et al. Elotuzumab plus lenalidomide/dexamethasone in relapsed or refractory multiple myeloma: extended 4-year follow-up and analysis of relative Progressione-Free Survival from the randomized ELOQUENT-2 trial. Cancer 2018; 124: 4032-43.
17. Dingli D, Ailawadhi S, Bergsagel PL, et al. Therapy for relapsed multiple myeloma: Guidelines from the Mayo stratification for myeloma and risk-adapted therapy. Mayo Clin Proc 2017; 92: 578-98.
18. Palumbo A, Bringhen S, Mateos MV et al. Geriatric assessment predicts survival and toxicities in elderly myeloma patients: an International Myeloma Working Group report. Blood 2015; 125: 2068-74.
19. Ludwig H, Delforge M, Facon T, et al. Prevention and management of adverse events of novel agents in multiple myeloma: a consensus of the European Myeloma Network. Leukemia 2018; 32: 1542-60.
20. Dimopoulos M, Facon T, Richardson P, et al. ELOQUENT-1: a phase III, randomized, open-label trial of lenalidomide/dexamethasone with or without elotuzumab in subjects with previously untreated multiple myeloma (CA204-006). J Clin Oncol 2012; 30 (15 Suppl) [abstract TPS8113].
21. Usmani SZ, Sexton R, Ailawadhi S, et al. SWOG 1211: initial report on phase I trial of RVD-elotuzumab for newly diagnosed high risk multiple myeloma (HRMM). In: 56th annual meeting and exposition of the American Society of Hematology; 2014; San Francisco, CA. [abstract 4762; poster]
22. Thomas SK, Shah JJ, Lee HC, et al. Preliminary results of a Phase II study of lenalidomide-elotuzumab as maintenance therapy post-autologous stem cell transplant in patients with multiple myeloma. Blood 2017; 130: 840.
23. Jagannath S,Laubach J,Wong E, et al. Elotuzumab monotherapy in patients with smouldering multiple myeloma. A phase II study. Br J Haematol 2018; 182: 495-503.
24. Ghobrial IM, Badros AZ, Vredenburgh JJ, et al. Phase II trial of combination of elotuzumab, lenalidomide and dexamethasone in high-risk smoldering multiple myeloma. Blood 2016; 128: 976.
25. Mateos MV, Granell M, Rocafiguera AO, et al. A phase II single arm safety study of elotuzumab in combination with thalidomide and low dose dexamethasone in patients with relapsed and/or refractory multiple myeloma. Haematologica 2014;99(Suppl 1) [abstract P959].

Correspondence:
Monica Galli, MD, PhD
Hematology & Bone Marrow Transplant Unit
ASST Papa Giovanni XXIII
P.zza OMS, 1 - 24127 Bergamo, Italy
Fax: 035 267 4968
E-mail: monicagalli@asst-pg23.it

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 80-85, 2018 © Mattioli 1885

Control data on endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Ramazzini Institute colony

Fabiana Manservisi[1,2], Laura Falcioni[1], Luciano Bua[1], Ilaria Menghetti[1], Daniele Mandrioli[1,3], Giovanna Galeati[2], Marcella Spinaci[2], Carlo Tamanini[2], Fiorella Belpoggi[1]

[1] Cesare Maltoni Cancer Research Center, Ramazzini Institute, Bentivoglio, Bologna, Italy; [2] Department of Veterinary Medical Sciences, University of Bologna, Italy, [3] Department of Agricultural Sciences, University of Bologna, Italy

Summary. *Background and aim:* Findings from laboratory animals as well as human studies suggest that Endocrine Disrupting Chemicals (EDCs) cause a number of reproductive health outcomes. Rats have been used extensively for developmental and reproductive physiology and endocrinology research and a number of endocrine sensitive endpoints have been well established in a variety of regulatory guidelines on rodent bioassays. We monitored the background data on some endocrine sensitive endpoints for untreated Sprague-Dawley rats from the Cesare Maltoni Cancer Research of the Ramazzini Institute colony (SD-CMCRC/RI). *Materials and methods:* General reproductive indices from dams and data for the entire litter were recorded. All the littermates were retained until the achievement of puberty and balanopreputial separation (BPS) was monitored in all the males; estrous cycle length and pattern were also evaluated in one female/litter. We compared our data with those provided by the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI). *Results:* Overall, reproductive indices and pre-post weaning litter data of SD-CMCRC/RI rats were comparable with those reported by ILSI. *Conclusions:* Procedures for monitoring and physiological biological variations in our SD-CMCRC/RI rats fall within the range of values typically obtained for the selected endpoints. Further investigations are suggested in order to verify whether retaining all pups to sexual maturation can improve the sensitivity to discriminate between natural variation and treatment effects. A more comprehensive analysis of other relevant endocrine sensitive endpoints should be performed in order to provide a representation of the normal developmental landmarks and endocrine values at different ages.

Key words: endocrine endpoints, historical control data, Sprague-Dawley rats

Introduction

Findings from laboratory animals as well as human studies suggest that Endocrine Disrupting Chemicals (EDCs) cause a number of reproductive health outcomes, including abnormal puberty, irregular estrous cycle, reduced semen quality, testicular dysgenesis syndrome and other adverse effects involving disruption of the Hypothalamus-Pituitary-Gonadal (HPG) and/or Hypothalamus-Pituitary-Thyroid (HPT) axis (1).

The laboratory rat is widely used as the traditional animal model of choice for research on developmental and reproductive toxicity testing, conducted to support human health hazard identification and risk assessment. Considering the substantial conservation of reproductive process across rat and human, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended the laboratory rat as the species of choice for the endocrine screening and testing assays (2, 3).

A number of endocrine-related endpoints has been well established in a variety of regulatory guidelines on rodent bioassays, including areola/nipple retention and anogenital distance in pups at birth; balano-preputial separation (BPS) in males and day of vaginal opening (VO) in females as primary landmarks of sexual development. Information on the integrity and performance of both male and female reproductive systems, including gonadal function, estrous cycle, mating behavior, conception, gestation, lactation, and the growth and development of the offspring are also addressed by current test methods focusing on developmental and reproductive toxicity. All these endpoints, sensitive to endocrine disruption, are well recorded in many Organization for Economic Co-operation and Development Test Guidelines (OECD TGs) such as the Two-Generation Reproduction Study (OECD TG 416) (4), the Extended One-Generation Reproduction Study (OECD TG 443) (5) and the Developmental Neurotoxicity (DNT) study (OECD TG 426) (6). The National Toxicology Program (NTP) has also developed a range of techniques and testing regimes to evaluate the potential of environmental and occupational substances to affect development and damage reproductive systems. The NTP's Modified One-Generation Reproductive study design (MOG) provides information on the effects of substances on prenatal development, postnatal development, and reproduction (7). Recently, many OECD TGs have been revised placing additional emphasis on endocrine endpoints; the need for careful clinical observations of the animals, so that to obtain as much information as possible, is also stressed (8, 9).

The use of rodent models for research and testing on EDCs needs an awareness of a number of laboratory animal science issues in order to standardize methods of monitoring thus facilitating the reproducibility of results among laboratories (10).

We monitored untreated Sprague-Dawley (SD) rats belonging to the colony of the Cesare Maltoni Cancer Research Center of the Ramazzini Institute (CMCRC/RI) in order to provide background data on some endocrine sensitive endpoints for interpreting experimental results in developmental/reproductive studies.

General reproductive indices of the dams were recorded for subsequent interpretation of reproductive health effects of a tested substance. Indeed, changes in reproductive indices can be due to several factors, including alteration in hormone levels and fetal growth retardation (11).

Data for the entire litter, including litter size and sex ratio, were reported as an important endpoint in the overall evaluation of reproductive performance. A decreased litter size may indicate an adverse reproductive effect and can be used as a nonspecific indicator of reproductive toxicity. Altered sex ratios may be related to several factors, including selective loss of male or female offspring, sex-linked lethality (genetic germ cell abnormalities), abnormal production of X or Y chromosome-bearing sperm, or hormonal alterations that result in intersex conditions (masculinized females or feminized males) (11).

All the littermates were retained until the achievement of puberty without performing culling (reduction of litter size) a widely used procedure in reproductive toxicity studies (12). The BPS was monitored in males. Cleavage of the balano-preputial gland is an apical measure of the progression of puberty and it has been used as the primary endpoint of puberty onset in the male rat as it is an androgen dependent event (13, 14).

In one female per litter, the estrous cycle pattern was determined by observing changes in the vaginal smear cytology. Vaginal cytology is known to be dependent upon the hormonal balance and to respond rapidly to the administration of chemical possessing hormonal activity as, for example, oestrogenic agonist or antagonist activity. The inclusion of an assessment of estrous cyclicity, by examination of vaginal smears or washes, offers a quick and easy way to measure the sex hormone status within the female and is of value in interpreting other findings (for example, weight or pathological data for the female reproductive organs). Potentially this technique could also act as a simple initial marker of changing reproductive capacity with age in chronic studies (11).

A comparison with the laboratory's historical control data is an important aid to determine whether small increases or decreases (including not statistically significant ones) in an endpoint might constitute a treatment-related effect. As part of this evaluation, we compared our data with those provided by the Health

and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI) that provided a retrospective analysis of 43 multi-generation studies (16 in Wistar rats, 27 in Sprague-Dawley rats) conducted according to the United States Environmental Protection Agency (U.S. EPA) Reproduction and Fertility Effects Test Guideline (OPPTS 870.3800/OECD 416) (15).

Materials and methods

Male and female SD rats belonging to the colony used in the laboratory of the CMCRC/RI for over 40 years were used in the experiment. All the animals were kept in a single room at 23±3°C and at 40-60% relative humidity. The light/dark cycle was 12 hours. Rat feed (Dr. Piccioni Laboratory, Milan, Italy) and tap water were available *ad libitum*. Each lot of feed and tap water was periodically analyzed for biological (bacteria) and chemical (mycotoxins, pesticides, arsenic, lead, mercury, selenium) contaminants.

Eleven virgin female rats were cohabited with 11 breeder male rats of the same strain, one male per female, never brother and sister. Every day, the females were examined for presence of sperm by vaginal cytology. The day in which sperm was found in vaginal canal was defined as Day 0 of pregnancy (GD 0). The fertility index was defined as the number of animals inducing pregnancy or becoming pregnant divided by the number of mating sets. The gestation index was reported as the percentage of pairs with confirmed mating that have produced at least one pregnancy within a fixed period. Mean gestational length (duration of pregnancy) was the time from GD 0 to parturition. The day birth occurred was designated as post natal day 1 (PND). Each dam and delivered litter were housed in a common nesting box during the postpartum period. Newborns were housed with their mothers until weaning at PND 28. Sex was determined on PND 1 and sex ratio data was presented as percentage of males to total number of offspring. The mean litter size, including dead as well as live offspring, was calculated on PND 1. We totally evaluated 136 pups, 67 males and 69 females.

All the littermates were observed until the achievement of sexual maturity.

Starting on PND 35 until completion, all the males were examined daily (between 9:00 A.M. and 12:00 P.M.) for BPS. Each male rodent was removed from its cage and held in a supine position. Gentle digital pressure was applied to the sides of the prepuce, and the criterion was met when the prepuce completely retracts from the head of the penis. Each male rodent was examined daily until acquisition.

Starting from young adulthood (approximately PND 120) and for the duration of 3 weeks, daily vaginal lavage was performed on one female/litter. The female rat was removed from the cage and approximately 0.25 ml of physiological saline solution were drawn into a new clean dropping pipette. The tip of the pipette was gently inserted into the vaginal canal, the pipette bulb was firmly but gently depressed to expel the saline into the vagina and the saline was drawn back into the dropping pipette which was removed from the vaginal canal. A spray fixative (Cytofix™ Fixation Buffer, BD Biosciences, supplied by Di Giovanni srl, Bologna, Italy) was applied onto the slide prior to Papanicolaou stain. By using Papanicolaou staining, the maturity of nucleated epithelial cells can be distinguished with less mature cells stained turquoise and more mature cells pink- or orange-stained. Briefly, slides were successively submerged in alcohol 95%, 80%, 70% and water, then stained with Harris' Hematoxylin solution (Labochimicha srl, Padua, Italy). After a brief dipping in diluted hydrochloric acid and water to remove excess stain, the cells were dehydrated prior to immersion in the Orange G (Labochimicha srl,), an alcohol based cytoplasmic counterstain which stains keratin in brilliant orange. Slides were raised off in 95% alcohol and stained with the second counterstain, Eosin-Azure (E.A.) 50 (Labochimicha srl,), and rinsed off in 95% again. Finally, slides were immersed in absolute alcohol to dehydrate completely and in xylene. Slides were mounted with the Permount, then coverslipped and observed under a light microscope. The cytology of the vaginal smears allowed a classification in the following estrous stages: diestrus (D), predominance of leukocytes and a few scattered cornified epithelial cells; proestrus (P), predominance of round nucleated epithelial cells that may be dispersed or clumped; or estrus (E), all cornified cells (16). All the vaginal smear slides were evaluated by two pathologists in blind and

any discrepancy was solved by final consensus. For each female, measurement of estrous cycle length was performed by selecting the estrous stage and counting until the recurrence of the same stage. An analysis of estrous cycle pattern was also performed and reported as percentage of time in each stage.

Results

Results for dams and pre-weaning pups of SD-CMCRC/RI rats are reported in Table 1. The female's ability to achieve pregnancy, calculated as fertility index, turned out to be 91.6%. All the pregnant dams maintained pregnancy and delivered live pups (gestational index equal to 100%). The eleven dams displayed a similar gestational length (22.9±0.8 days). The mean litter size was 12.4±2.2 and sex ratio at birth (% males/total offspring) was 48.5±9.8.

Data on post-weaning endpoints are presented in Table 2. Balanopreputial separation, evaluated in all the littermates, was achieved at PND 45.0±1.9. The mean estrous cycle length, evaluated in one female/litter, was 4.9±0.3 days. Estrous cycle pattern, evaluated over a 3-week monitoring period, revealed a percentage of 51.4±9.2 days in diestrus; 24.8±6.3 in proestrus and 23.8±4.5 in estrus. The comparison of dams and pre-post weaning data of pups between SD-CMCRC/RI rats and inter-Laboratory control SD- derived rats data provided by ILSI is reported in Table 3.

Table 1. Dams and pre-weaning litter data from SD-CMCRC/RI rats.

Parameter	SD- CMCRC/RI
Fertility index (%)[a]	91.6
Gestational index (%)[b]	100 (11/11)
Mean gestational length (day) [c,d]	22.9±0.8
Total pups (n) delivered at PND 1 [e]	136
Litter size (n) [d,f]	12.4±2.2
Total male pups (n) at PND 1	67
Total female pups (n) at PND 1	69
Sex ratio at birth (%) [d,g]	48.5±9.8

[a:] Fertility index = (number of pregnant females/number of females cohabitated) x 100
[b:] Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100
[c:] Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[d:] Mean ± standard deviation
[e:] Live and stillborn pups are considered
[f:] Mean number of pups per litter at PND 1 (within 24 hours from delivery)
[g:] Sex ratio at birth = (no. of male offspring/no. of total offspring) x 100

Table 2. Post-weaning landmarks of pups from SD-CMCRC/RI rats.

Parameter	SD- CMCRC/RI
Age (PND) at balano-preputial separation (BPS) [a]	45.0±1.9
Estrous cycle length (days) [a]	4.9±0.3
Time in diestrus (%)[a]	51.4±9.2
Time in proestrus (%)[a]	24.8±6.3
Time in estrus (%)[a]	23.8±4.5

[a:] Mean standard deviation

Table 3. Comparison of dams and pre-post weaning data of pups between SD-CMCRC/RI rats and SD-derived rats*.

Parameter	SD- CMCRC/RI	SD-derived*
Fertility index (%)[a, b]	91.6	89.8±5.9
Gestational index (%)[b, c]	100	99.2±2.6
Mean gestational length (day) [b, d]	22.9±0.8	22.1±0.4
Litter size (n) [b, e]	12.4±2.2	13.7±0.9
Sex ratio at birth (%) [b, f]	48.5±9.8	52
Age (PND) at balano-preputial separation (BPS)[b]	45.0±1.9	45.3±2.1
Estrous cycle length (days) [b]	4.9±0.3	4.2±0.4

* Crl:CD®(SD)IGS BR, Crl:CD® (SD)IGS BR–VAF/Pluss, Crl:CD (SD), Crl:CD® (SD) BR, Crl:CD® BR, Crl:CD® BR–VAF/Pluss, CD®
[a]: Fertility index = (number of pregnant females / number of females cohabitated) x 100
[b:] Mean ± standard deviation
[c:] Gestational index = (number of females with live born / number of females with evidence of pregnancy) x 100
[d:] Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[e:] Mean number of pups per litter at PND 0 (within 24 hours from delivery)
[f]: Sex ratio at birth= (no. of male offspring/no. of total offspring) x 100. Standard deviation for control values is not reported by Marty MS et al. 2009

Discussion

Comprehensive historical control data are important in toxicity studies, as comparisons of data from study controls with historical ones may help to distinguish treatment-induced changes from spontaneously occurring background changes specific to species and strains (17).

Caution should be taken particularly when comparing certain endpoints, such as endocrine-related endpoints, with historical control databases from other laboratories, owing to possible inter-laboratory differences in procedures and classification schemes. Furthermore, subtle changes in species occur over time, owing to genetic alterations in strains or stocks of species and to change in environmental conditions, both in breeding colonies and in individual laboratories (17).

In our work, the reproductive indices and preweaning litter data of SD-CMCRC/RI rats were comparable with those reported by ILSI.

Interestingly, for SD-derived rats, data were separated by ILSI into litters that were standardized (i.e., culled) or not. In our work, pups were not culled, all the littermates were retained until the time of puberty. Culling is a procedure of artificial equalization of the number of offspring in litter used in rodent experiments to control litter size (18). The rationale for unculling litters is based on the possibility to explore the litter variability and to improve the sensitivity of the statistical analysis in detection of statistically significant and biologically important differences in maturational endpoints. Further statistical analysis on individual data from different laboratories could help to demonstrate whether culling evaluation of all the littermates *vs* one or two pups/sex/litter influences the outcome of data by reducing the probability of identifying a false negative result.

We also evaluated some endocrine relevant endpoints that are currently required by the OECD TGs, i.e BPS in males and estrous cyclicity in females.

The mean ages at BPS in SD-derived rat, reported by ILSI, ranged between 41.2 and 49.0 days, with a mean of 45.0±1.9 days. These values were remarkably closed to those obtained by the SD-CMCRC/RI male rats (45.3±2.1), indicating that the assessment was conducted in a consistent manner within and between studies. It is also noteworthy that BPS was monitored by the examination of all littermates. This procedure represents a new and interesting perspective, indeed, sexual maturation assessments are usually performed on only one weanling rat per sex after litter standardization or culling. For these reasons, the reported historical control values are usually based on observations for one weanling pup/sex/litter. Concern is expressed that culling could affect many health-related endpoints, including the onset of developmental landmarks and sexual maturation (12, 18).

In females, estrous cycle data are used to complement other data and do not typically indicate an adverse effect alone. Cooper and Goldman (19) reported that estrous cycle pattern is an important parameter in order to detect changes that might be masked when only examining estrous cycle length. Altered estrous cyclicity or complete cessation of vaginal cycling in response to toxicants should be considered an adverse female reproductive effect. In our work, data on estrous cycle length were within the expected range (e.g., 4-5 days). While estrous cycle length was reported in the ILSI review, estrous cycle pattern was not included due to the lack of the evaluation or of an agreed-upon method for correctly assessing cycle normality and duration (15). Consequently, a comparison for this endpoint was not possible.

Conclusions

Overall, the data for endocrine sensitive endpoints from our untreated SD-CMCRC/RI rats are comparable to the value reported in the scientific literature, suggesting that procedures for monitoring and physiological biological variations in our SD rats fall within the range of values typically obtained for the selected endpoints. In particular, BPS values were comparable for unculled SD-CMCRC/RI rats and other culled SD-derived rats. Further data on other relevant endocrine sensitive endpoints, such as anogenital distance, vaginal opening, first estrus and relative body weight at the time of acquisition, sperm analysis need to be investigated in our SD colony in the future, in order to provide a more comprehensive perspective

for interpreting data from treated animals, particularly with regard to reproductive and developmental toxicity bioassays.

Author contribution:
Fabiana Manservisi, Laura Falcioni, Luciano Bua, Ilaria Menghetti: concept and design of study, data collection, data interpretation and analysis, drafting, revision, approval of final manuscript; Daniele Mandrioli, Giovanna Galeati, Marcella Spinaci, Carlo Tamanini and Fiorella Belpoggi: data interpretation and analysis, critical revision of the entire text, approval of final manuscript.

References

1. Manibusan MK, Touart LW. A comprehensive review of regulatory test methods for endocrine adverse health effects. Critical Reviews in Toxicology 2017; 47: 440-488.
2. Gray LE, Jr., Wilson V, Noriega N, et al. Use of the laboratory rat as a model in endocrine disruptor screening and testing. ILAR J 2004; 45: 425-437.
3. Manservisi F, Marquillas CB, Buscaroli A, et al. An Integrated Experimental Design for the Assessment of Multiple Toxicological End Points in Rat Bioassays. Environ Health Perspect 2017; 125: 289-295.
4. OECD. Test No. 416: Two-Generation Reproduction Toxicity. 2001.
5. OECD. Test No. 443: Extended One-Generation Reproductive Toxicity Study. 2018.
6. OECD. Test No. 426: Developmental Neurotoxicity Study. 2007.
7. NTP. NTP's Modified One-Generation Reproduction Study. 2011.
8. OECD. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. 2018.
9. OECD. Test No. 414: Prenatal Developmental Toxicity Study. 2018.
10. Everitt JI, Foster PMD. Laboratory Animal Science Issues in the Design and Conduct of Studies with Endocrine-active Compounds. ILAR Journal 2004; 45: 417-424.
11. Hood R. Developmental and reproductive toxicology - a practical approach. 2006.
12. Palmer AK, Ulbrich BC. The cult of culling. Fundam Appl Toxicol 1997; 38: 7-22.
13. Lyons W. R. BI, Friedlander S. Cornification of balano-preputial epithelium in normal rats and in castrated rats treated withm testosterone propionate. Endocrinology 1942; 31: 659-663.
14. OECD. Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. Series on Testing and Assessment. No. 150. Paris : OECD Publishing, 2014.
15. Marty MS, Allen B, Chapin RE et al. Inter-laboratory control data for reproductive endpoints required in the OPPTS 870.3800/OECD 416 reproduction and fertility test. Birth Defects Res B Dev Reprod Toxicol 2009; 86: 470-489.
16. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res B Dev Reprod Toxicol 2007; 80: 84-97.
17. Kuwagata M, Sakai Y, Tanaka S, et al. Historical control data on developmental toxicity studies in rats. Congenit Anom (Kyoto) 2018 [Epub ahead of print].
18. Suvorov A, Vandenberg LN. To Cull or Not To Cull? Considerations for Studies of Endocrine-Disrupting Chemicals. Endocrinology 2016; 157: 2586-2594.
19. Cooper RL GJ. Vaginal cytology. In Daston G KC (ed) An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington, DC: ILSI Press. 1999; p 42-56.

Correspondence:
Fabiana Manservisi,
Cesare Maltoni Cancer Research Center, Ramazzini Institute
Via Saliceto 3, 40010 Bentivoglio, Bologna, Italy
Tel. +39 051 6640460
Fax +39 051 6640223
E-mail: manservisif@ramazzini.it

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 86-98, 2018 © Mattioli 1885

The analysis of longitudinal data from life-span carcinogenicity bioassays on Sprague-Dawley rats

Daria Sgargi[1], Simona Panzacchi[1], Daniele Mandrioli[1], Fiorella Belpoggi[1], Rossella Miglio[2]

[1] Cesare Maltoni Cancer Research Center, Ramazzini Institute, Bentivoglio 40010, Bologna, Italy; [2] Department of Statistical Sciences, University of Bologna, Italy

Summary. *Background and aim of the work:* Long Term Carcinogenicity Bioassays (LTCB) are among the best instruments to strengthen the evidence on which regulatory agencies base their decision to classify harmful agents as human carcinogens, so they are fundamental to protect public health. The statistical analysis is essential to validate the results from cancer and non-cancer outcomes in carcinogenicity bioassay. This work proposes and applies some methodologies for the analysis of non-cancer outcomes, such as body weights. *Methods:* We use data from studies already concluded, evaluated and published: 4 bioassays aimed at testing the carcinogenic potential of Coca-Cola on Sprague-Dawley rats of different ages. The analysis of body weights of the second generation of rats was performed using mixed-effects models: linear models were fitted for nonlinear models we considered human non-linear growth functions. *Results:* Linear models were fitted using the log-transformation of time and polynomial term of third order for time. Sex and treatment influence body weight, age of dams during gestation doesn't. Growth models: Jenns-Bayley, Count and 1st order Berkey-Reed growth functions were evaluated; the latter best describes the data. Sex and treatment significantly influence all parameters. The direction, magnitude and significance of the effect variable is substantially similar in all models. The analysis of residuals highlights the same issues for all models: the extreme trends in the last part of life heavily affect the models' performance. *Conclusions:* Mixed-effects models allowed to account for the structural effect of covariates that act the same way on all individuals, and to add random effects that introduce a correlation among subjects if clustering happens; nonlinear human growth models added information about the whole growth process, therefore these may be useful methods in studies focused on development and sexual maturation.

Key words: longitudinal analysis, body weights, Sprague-Dawley rats, mixed-effects models, carcinogenicity studies

Introduction

Cancer is a major issue of public health and despite the progress achieved in the prevention and cure of the disease, it is still the second leading cause of death worldwide. The prevalence of risk factors is in fact increasing, including occupational, environmental factors or consisting of dangerous behaviours and lifestyles, such as pollution, smoking, alcohol consumption, obesity and hypertension (2, 3). In this framework, the importance of primary prevention is clear: in terms of public health, the experimental research on environmental and occupational agents is fundamental in order to identify carcinogens and give to national

and international public health agencies adequate data for the necessary regulation.

Epidemiological and experimental studies are the best source of evidence to identify the carcinogenic hazard of a substance and quantify the risk linked to exposure. The most predictive experimental model to anticipate human carcinogens are long term and life-span carcinogenicity bioassays (4-6).

The importance of the statistical analysis of the data obtained through long term studies is generally recognised: it is the necessary complement to establish and quantify whether the long term exposure to selected agents is associated with adverse effects, and it should always be regarded as an integral part of the studies (7). Despite this, not all guidelines explicitly treat in detail and depth the statistical analysis of data (Hothorn, 2014). Different guidelines, mostly from the Organization for Economic Cooperation and Development, illustrate and explain how to choose and perform the appropriate statistical tests, based on the kind of experiment, its objectives and the type of data; they also help to interpret the results and to understand their real meaning and relative importance (3, 5). To maintain coherence with the established methods in toxicology, the classical frequentist approach and the concept of hypothesis testing are adopted; the methods are systematically organized into a flowchart, proposing tests to verify the significance of differences between the treated and the control groups, according to the nature of the data. Consolidate and advanced methodologies exist for the direct assessment of carcinogenicity, specifically developed to handle peculiarities of these data and answer specific research questions. However, additional information that are routinely collected in experimental studies (such as body weights, feed and fluid consumption, the time of survival in life-span studies, etc…) are only used to monitor the conduct of the study and the health status of animals; no specific statistical method to treat them is suggested, so they are rarely analysed in depth.

Many methodologies nowadays exist to analyse these data, and are relatively easy to implement, thanks to different accessible statistical software: applying them would allow to fully use all the available data and to integrate them, reaching an overall more complete information on the effects of the tested compound on health.

The aim of this work is to better exploit the potential of all available data on non-cancer outcomes from carcinogenicity studies to strengthen the knowledge of the tested substances. In particular, the objectives of this study are to examine one of the most common type of non-cancer outcomes, the body weight of experimental animals, to find appropriate methodologies to analyse its characteristics, and to apply them on some real data, in order to verify their suitability.

Materials and Methods

The data for this analysis were obtained from studies performed in 1986 at the Cesare Maltoni Cancer Research Centre (CMCRC) of the Ramazzini Institute, aimed at evaluating the possible association between continuous consumption of Coca-Cola and effects on tumour incidence in rodents. The soft drink was chosen as a test substance because of its widespread diffusion, the known effects of sweetened beverages on weight and the growing awareness of the importance of obesity as a risk factor for several types of tumours.

Four experiments were performed, each involving male and female Sprague-Dawley rats starting exposure at different ages (breeding rats of 30, 39 and 55 weeks of age; all their offspring of all litters, whose observation started at 8 weeks of age; and young non-breeding rats of 7 weeks of age). The experimental plan is schematically reported in Table 1. The soft drink was administered to rats ad libitum as a substitute of drinking water from the beginning of observation for the whole lifespan, until spontaneous death.

Here, we will focus on the second generation of rats: treated female and male breeders started to drink Coca-Cola one week before mating, while the control group was administered with tap water; dams continued the exposure during the whole period of the pregnancy and the weaning. After weaning, offspring continued to drink Coca-Cola ad libitum and from 8 weeks of age they were weighted and controlled for feed and beverages consumption until spontaneous death. All pups from all litters were included, in the same experimental group as their breeders, so rand-

Table 1. Experimental plan of the four bioassays performed for the project: treatments, age at beginning of observation and number of animals by sex for each experimental group.

Treatment	Age at start	M	F
Coca-Cola	7 weeks	80	80
Drinking water	7 weeks	100	100
Coca-Cola	55 weeks	70	70
Drinking water	55 weeks	70	70
Coca-Cola	Prenatal (offspring)	28	24
Drinking water	Prenatal (offspring)	32	24
Coca-Cola	30 weeks	55	55
Drinking water	30 weeks	55	55
Coca-Cola	Prenatal (offspring)	74	73
Drinking water	Prenatal (offspring)	110	98
Coca-Cola	39 weeks	110	110
Drinking water	39 weeks	110	110
Coca-Cola	Prenatal (offspring)	67	65
Drinking water	Prenatal (offspring)	49	55

omization was not used for the second generation; the data can therefore be considered clustered.

The experiments were planned and performed following the standard procedures of the CMCRC and in compliance with international guidelines; for a detailed presentation of the experimental plan, conduct and results of the analysis of tumour incidences, see the original publication from Belpoggi et al. (8).

This work is focused on the analysis of longitudinal measurements of body weight, one of the best indicator for the good conduct of chronic rodent bioassays; moreover for this particular test compound, a sugar-sweetened beverage, the body weight is an endpoint of particular interest, as well as a very important indicator of metabolic, hormonal and homeostatic functions, growth and sexual maturation (9).

Guidelines (5) suggest to graphically represent groups means to keep track of the indicatiors of the animals' well-being during the experiments; then, formal analysis should start checking the assumptions of normality, homogeneity of variance and absence of outliers, required for the subsequent analyses; in case the assumptions are not met, some solutions such as the log-transformation of data are suggested. Finally, several types of tests are proposed, to evaluate the differences between groups (Student's t-test or modified t-test with Satterthwaite's method, or ANOVA and pairwise comparisons). A concise representation of the suggested analyses can be seen in Figure 1.

This approach has some clear drawbacks: using overall summary measures instead of all available data for each individual in time may cause a loss of information. Furthermore, it is impossible to evaluate the effect of more variables at a time.

The methods that might be more suitable for analysing this kind of data (clustered longitudinal data) are based on mixed-effect models. Laird and Ware (10) were the first to propose a flexible class of mixed models for longitudinal data: it includes both growth and repeated-measures models as special cases, and it introduces population parameters, individual effects and within-subject variation, as well as between-subject variation (11, 12).

In their representation, the $n * 1$ vector of responses for the ith subject can be modelled as

$$\boldsymbol{y}_i = \boldsymbol{X}_i\boldsymbol{\beta} + \boldsymbol{Z}_i\boldsymbol{b}_i + \boldsymbol{\varepsilon}_i \qquad i=1,\ldots N$$

where

- $\boldsymbol{X}_i$ is a $n_i * p$ design matrix of explanatory variables or fixed factors;
- $\boldsymbol{\beta}$ is a $p * 1$ vector of unknown population parameters, or fixed effects coefficients, describing the relationships between the outcome and the explanatory variables for groups defined by levels of a fixed factor (for example, describing the contrast between males and females);
- $\boldsymbol{Z}_i$ is a $n_i * q$ design matrix of variables of random factors;
- $\boldsymbol{b}_i$ is $q * 1$ a vector of unknown random effects specifically referred to a given level of a random factor, usually representing the deviations from the relationships described by fixed effects. Random effects can be set as random intercepts (random deviations for an individual or cluster from the overall fixed intercept), or as random coefficients (random deviations for an individual or cluster from the overall fixed effects). They are assumed to follow a multivariate normal distribution ~ $N(\mathbf{O}, \boldsymbol{D})$ with D being a $q * q$ symmetric, positive definite variance-covariance matrix;
- $\boldsymbol{\varepsilon}_i$ is a $n_i * 1$ vector of errors for the ith subject for each measurement occasion, whose terms do not

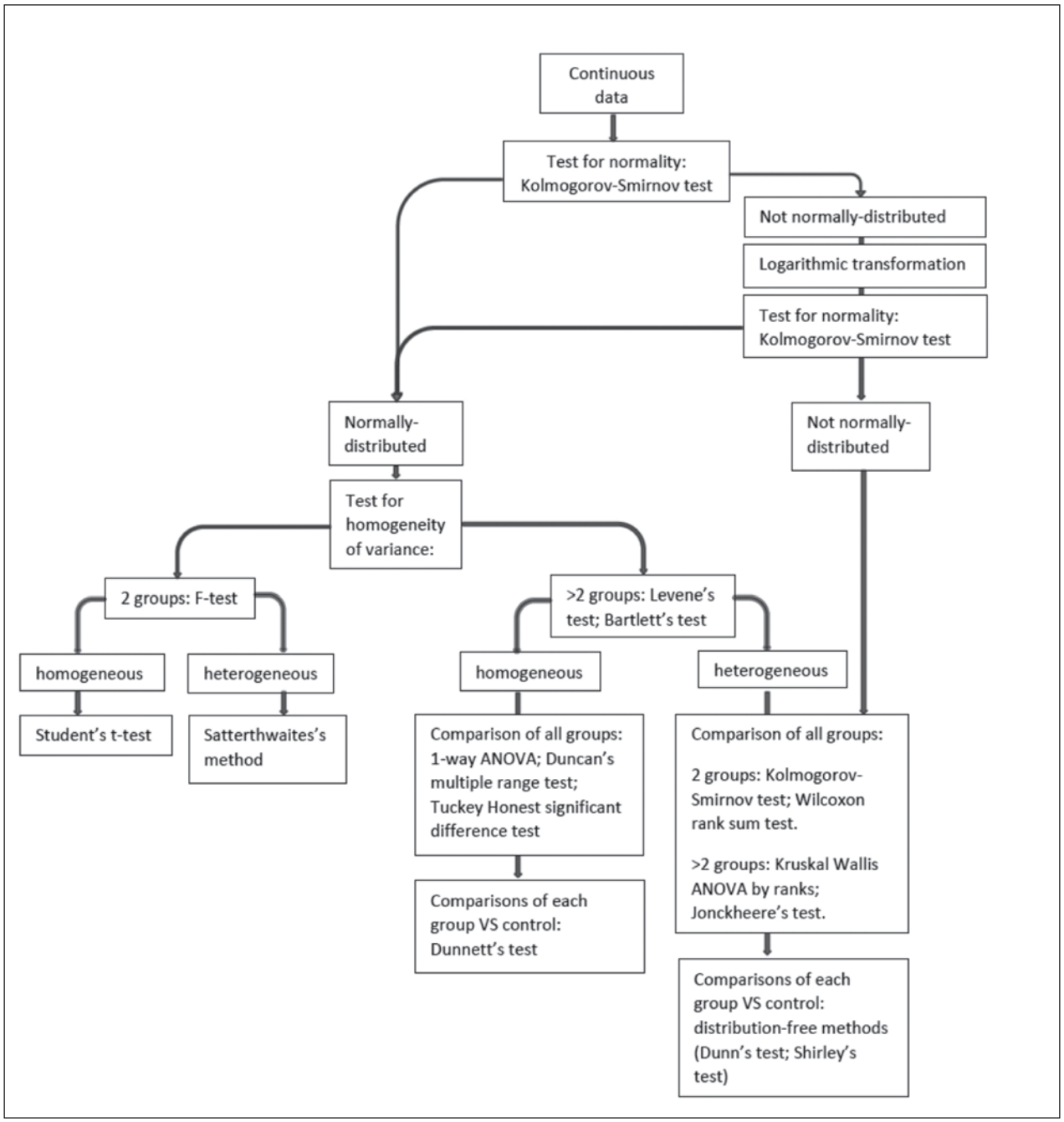

Figure 1. OECD statistical decision tree summarizing the suggested procedures for the analysis of continuous data. (Author's adaptation from OECD, 2012)

need to be independent but can be correlated within individuals. The residuals for each subject follow, again, a multivariate normal distribution ~ $N(\boldsymbol{O}, \boldsymbol{R}_i)$, with 0 mean and a positive definite $q * q$ variance-covariance matrix, $\boldsymbol{R}_i$.

Several covariance structures can be specified both for $\boldsymbol{D}$ and $\boldsymbol{R}_i$. Inference on the parameters' estimates can be based on least squares and maximum likelihood methods, or, formulating the model the appropriate way, using an empirical Bayesian method (13, 14).

The response variable is here assumed to present a linear and continuous trend, so the function included in the model to represent its relationship with independent variables through fixed and random effects is a linear one. Often, however, the trajectory of individual growth presents discontinuities or shows a nonlinear path. Several adaptations of the linear mixed effects model may be adopted to cope with these situations, the most common being:

- splitting the time of analysis into sub-periods, so that the linearity assumption is reasonable within each sub-model;
- identifying a suitable transformation of the outcome variable or the time scale;
- representing time as a polynomial function (15).

All these methods share a characteristic: they still imply a linear association that models the relationship between the outcome and the explicative variables. Often, however, the likelihood function depends on the parameters in a non-linear way: in such cases, the use of nonlinear models is justified by the possibility to obtain a more interpretable model and to use a smaller number of parameters.

Lindstrom and Bates (16) were the first to present a general, nonlinear mixed effects model for data in which the assumption of the normality of residual holds, but the expectation function is nonlinear. The model can be written as

$$y_{ij} = f(x_{ij}, \beta, u_i) + \varepsilon_{ij}, \qquad i = 1,\ldots,N$$

where f is a real-valued function x_{ij} is a vector of covariates containing both within- and between-subjects covariates, β is a $q * 1$ vector of unknown parameters of fixed effects, u_i is a vector of unobservable subjective random parameters following a multivariate normal distribution with 0 mean and variance-covariance matrix Σ, and ε_i is the error vector of dimension $n_i * 1$, following a multivariate normal distribution with 0 mean and variance-covariance matrix $\sigma^2 \Lambda$.

The two-stages representation of the model (17) helps to clarify how the non-linear function is used to express the individual trajectory of change at level 1

$$y_{ij} = m\left(x_{ij}^{w}, \varphi_i\right) + \varepsilon_{ij},$$

where m describes the behaviour of the individual growth as depending on individual-specific parameters φ_i and the vector of within-subject covariates x_{ij}^{w}, while the inter-individual variability can be expressed using a regular linear relationship at level 2:

$$\varphi_i = d\left(x_{ij}^{b}, \beta, u_i\right)$$

where d is a vector function that explains the variation of individual-specific parameters between subjects and incorporates β, the vector of parameters for the population, and x_{ij}^{b}, the set of between-subjects covariates. The assumptions underlying the non-linear mixed effects model are that the random effects u_i and the error terms ε_i are independent between each other and across individuals, that $\sigma^2 > 0$ and that matrix Σ is definite nonnegative.

Choosing the correct functional form to specify the relationships at level 1 is very important to obtain credible and accurate models. For this analysis a peculiar approach was experimented.

The growth and maturation processes have long been studied in humans, and several models have been proposed to formalize their patterns during infancy, childhood and adolescence (18). It is well established that the pattern of growth of body dimensions of the "general type" (to be distinguished from those of lymphoid, neural and genital type) from birth to the adult age is increasing and S-shaped, since it progresses rapidly in the first years, then slows down, and accelerates again around the so-called pubertal spurt, and finally approaches a plateau when the approximate adult size is reached. Since many similarities can be recognized in the growth path of humans and rats, some of these human models have been translated, adapted and applied here to rats.

Several structural regression models based on an adequate parametric function were developed in time to represent the different phases of growth: these functions can be substituted to the generic function at the level 1 of the multilevel mixed effect model, since they describe the behaviour of each individual.

The first parametric model was elaborated already in 1937 by Jenss and Bayley (19): it was developed to describe growth from birth to approximately 8 years using 4 parameters combined in a function with a lin-

ear and an exponential part, accounting for growth and its decreasing rate:

$$y = a + b\,t - - e^{c+d\,t}$$

Another option is the Count model (20) proposed in 1943, that uses only 3 parameters combined in a linear way

$$y = a + b\,t + c\,\ln(t+1)$$

This model proved to perform slightly worse than the Jenss and Bayley, but both remain robust relative to the choice of starting values for the parameters. The Count model was later modified by Berkey and Reed (21) maintaining the simple, linear structure but adding one or two parameters:

$$1^{st}\ order:\ y = a + b\,t + c\,\ln(t+1) + \frac{d}{t}$$

$$2^{nd}\ order:\ y = a + b\,t + c\,\ln(t+1) + \frac{d_1}{t} + \frac{d_2}{t^2}$$

accommodating for one or two additional inflexion points and leading to a better fit, compared to the previous alternatives.

More complex models were developed to represent different phases of growth at the same time. It was showed (22) that rat's and human developmental phases are similar but growth rhythms differ, particularly in early phases, so it was chosen not to consider the models that were designed to account for the specific features and mechanisms of human growth, but rather to focus on those that could be used to describe a similar path, in terms of intensity and velocity, to the one of humans during young age, and adapt them to the available data from rats.

Results

All statistical and graphic analysis have been performed using the statistical software StataIC 15.

It was chosen to consider for analysis only the measurements taken until 114 weeks of age of rats, because after this timepoint the number of rats alive was considerably reduced (at 114 weeks of age, 52.5% of the animals were lost to follow-up; at 122 weeks of age, the following measurement, 91.4% were lost), and rats can be considered very old at this age (it is not possible to draw exact parallels, but it is well accepted (23) that 104 weeks of age in rats are comparable to around 65 years in humans).

The graphical analysis of individual and mean weights showed a high variability, both within and among subjects. All trends were quite similar in shape during the first period of growth, while during the adult/elderly period some peculiar patterns appeared: weights tended to decrease in the last part of life, because of diseases or the physiologic ageing process; some animals, on the other hand, experienced a rapid increase of weight due to the onset of mammary neoplastic lumps.

Given these characteristics that prevent the use of linear functions *tout court*, three options were evaluated for analysis: estimating linear mixed-effects models (I) using a mathematical transformation for the time variable, (II) using polynomial functions to represent time, to obtain an approximately linearized growth trajectory for each individual, and (III) fitting mixed-effects model using nonlinear "human" growth functions at the individual level. In all cases, the variables reflecting experimental conditions (such as sex, treatment regimen, and age of the dam at the beginning of gestation) were evaluated as fixed effect, while random intercept and slopes depending on time were introduced at the litter level, allowing correlation between the random slopes and intercepts.

I. Linear mixed-effect model, mathematical transformtation of time variable

Sex and the treatment regimen were found to significantly influence body weights; males ware sensibly heavier than females since the first observation; the treatment was responsible of a smaller but still relevant (p= 0.021) increase in weight since the first assessment. These results are summarised in Table 2; Figures 2 and 3 show a graphical representation of the average growth trajectories for treated and control animals, in male and female rats, and some example of the specific trajectories estimated for randomly selected litters, respectively.

Residuals were analysed to verify whether the underlying basic assumptions (linearity of the relationship between the outcome and the regressors, normality and homoscedasticity of residuals) were met: the unpredictable variability in the last phase of life was, as expected, very high.

II. Linear mixed-effect model, polynomial representation of time

The best option to represent time in this context is a third order polynomial. Results regarding the direction and magnitude of the effect of each variable on body weight and its overall relevance, are substantially assimilable to those obtained in the previous analysis. Again, the unconstrained consumption of Coca-Cola was associated with a significant (p= 0.016) increase in body weight, as illustrated in Table 3 and in Figures 4 and 5. According to the goodness of fit measures, anyway, the previous specification should be preferred. The analysis of residuals highlighted again that the problems arising from the extreme values of weights of elderly or ill rats remained quite evident: residuals had some issues concerning normality, and appeared quite heteroscedastic in relation to time, too.

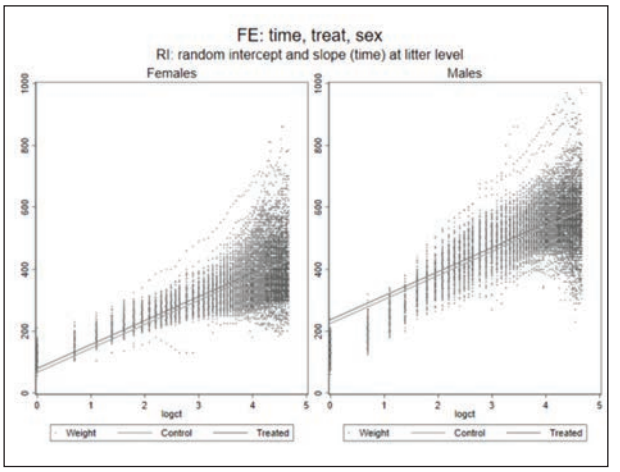

Figure 2. Plot of body weights (in grams) and estimated average predictions obtained using a linear multilevel mixed effects model (fixed-effects predictors: natural logarithm of time since first observation (in weeks), sex and treatment).

III. Nonlinear mixed-effects models, human growth functions

After comparing the performances of models built with the Jenns and Bayley, the Count and the 1st order Berkey and Reed growth functions, the Berkey

Table 2. Linear multilevel mixed effects model using transformed variables; results from the regression of body weights (in grams) on the natural logarithm of time since first observation (in weeks), sex and treatment

Mixed-effects REML regression of body weight					
Fixed-effects Parameters	Coef.	Std. Err.	P>|z|	[95% Conf. Interval]	
log(time)	77.69	1.98	0.000	73.81	81.57
Treatment	12.17	5.29	0.021	1.80	22.54
Sex	158.16	0.54	0.000	157.10	159.22
_constant	67.01	4.51	0.000	58.16	75.86
Random-effects Parameters	Estimate	Std. Err.		[95% Conf. Interval]	
Litter: Unstructured					
sd(log(time))	19.4	1.43		16.79	22.42
sd(_cons)	35.88	3.19		30.14	42.72
corr(log(time),_cons)	-0.71	0.06		-0.81	-0.57
sd(Residual)	47.55	0.18		47.21	47.9

Goodness-of-fit Measures			
Log-likelihood	-193723.6	*df*	8
AIC	387463.1		
BIC	387531.2		

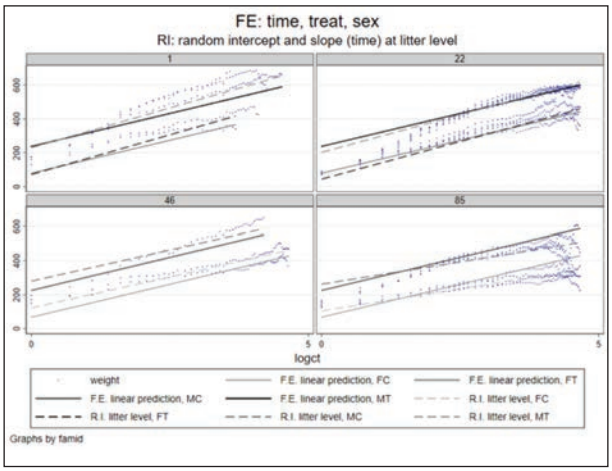

Figure 3. Plot of body weights (in grams) and linear predictions with fixed and random part in four randomly selected litters; results obtained using a linear multilevel mixed effects model (fixed-effects predictors: natural logarithm of time since first observation (in weeks), sex and treatment).

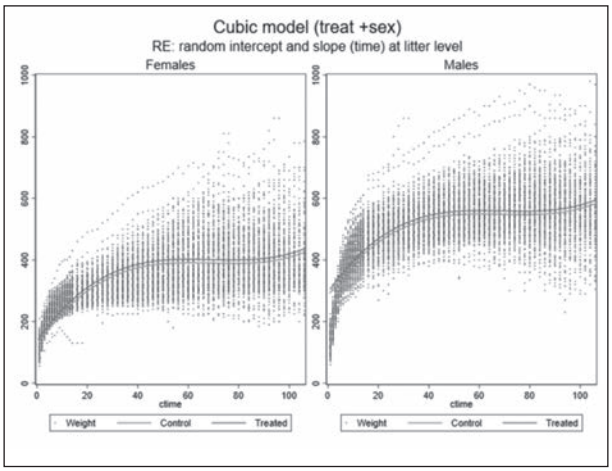

Figure 4. Plot of body weight (in grams) measurements and estimated average predictions, linear multilevel mixed effects model using a third degree polynomial term to represent time since first observation (in weeks)

Table 3. Linear multilevel mixed effects model using polynomial representation of time; results from the regression of weight (in grams) on third degree polynomial term for time since first observation (in weeks), sex and treatment.

Mixed-effects REML regression					
Fixed-effects Parameters	Coef.	Std. Err.	P>|z|	[95% Conf. Interval]	
Time	11.92	0.19	0.000	11.56	12.29
time^2	-0.17	0.00	0.000	-0.18	-0.17
time^3	0.00	0.00	0.000	0.00	0.00
Treatment	13.42	5.59	0.016	2.46	24.38
Sex	158.13	0.57	0.000	157.01	159.25
_constant	123.43	3.88	0.000	115.83	131.03
Random-effects Parameters	Estimate	Std. Err.		[95% Conf. Interval]	
Litter: Independent					
sd(time)	1.64	0.15		1.37	1.95
sd(time2)	0.03	0.003		0.02	0.03
sd(time3)	0.0002	0.00002		0.0001	0.0002
sd(_cons)	26.47	2.16		22.55	31.07
sd(Residual)	49.76	0.18		49.39	50.12

Goodness-of-fit Measures			
Log-likelihood	-195757.7	*df*	11
AIC	391537.4		
BIC	391631		

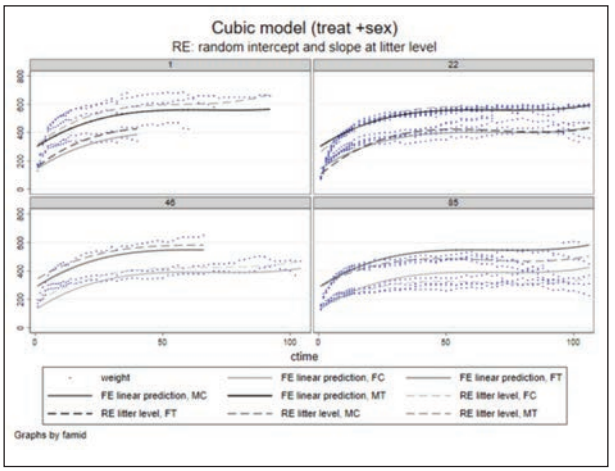

Figure 5. Plot of body weights (in grams) and linear predictions with fixed and random part in four randomly selected litters, linear multilevel mixed effects model using third degree polynomial to represent time since first observation (in weeks).

and Reed was chosen as the most appropriate for these data. The model building in this case was quite different from the previous ones: it uses four parameters to describe the specific functional form of the individual growth curve

$$y = a + b\,t + c\,\ln(t+1) + \frac{d}{t}$$

that may respectively represent the starting point, growth rate, acceleration and deceleration of growth. Here, the function was built so that each parameter would be dependent on sex and treatment regimen; a random effect at the litter level was introduced and evaluated for all of them, so that each litter is not constrained to have, for example, the same intercept or inflexion points. The estimated parameters are not as easy to interpret, as in the previous cases; we can resume, anyway, that the previous results are confirmed (the individual change depended on age, while the inter-individual change was described using sex and the treatment regimen as fixed effects, and a random effect at the litter level, to explain the variations in each parameter of the model). The estimates and their graphical representation are displayed in Table 4, and in Figures 6 and 7. Nevertheless, the problems of excessive variability in the tail of the growth trajectories remained relevant, as expected.

Discussion

The aim of this work was to go beyond the standard statistical techniques that are routinely used in experimental carcinogenicity studies to analyse non-cancer endpoints. We proposed and applied some methodologies that encompass the use of the different approaches, instead of summary measures, in order to answer the research questions in a more comprehensive way.

It is difficult to directly compare the results with those of the original publication, since different approaches were adopted. The use of mixed-effects models allowed to use every measurement available from each individual animal: this was important given the features of the data, that presented a consistent random variability, mostly in the last part of the animals' lives. Furthermore, it allowed to account for the structural effect of covariates that act the same way on all individuals, and to add random effects that introduce a correlation among subjects, accounting for clustered data. Finally, applying nonlinear human growth functions allowed to consider the change of body weight in time as a process, instead of a generic series of measurements: this can be useful in studies whose aim is to characterise possible variations in the development and the sexual maturation linked to the exposures under analysis.

In this study, where the tested compound was a highly caloric and sweetened beverage, body weights are of primary interest, and it would be even more interesting to deepen the analyses evaluating them in association with tumour incidence. Indeed, the unconstrained consumption of Coca-Cola in this experiment was associated with a significant increase in body weight (the statistically significant result emerges from all estimated models: linear mixed-effect model, logarithmic function of time: p= 0.021; linear mixed-effect model, cubic function of time: p= 0.016; non-linear mixed-effect model, Berkey-Reed growth function: p= 0.000 for all parameters) and it is well established that overweight and obesity are positively associated with the increase of the risk of many types of cancer (24-26). They were not explicitly considered here, but these analyses are suitable for rats observed from an adult age, as well; for this purpose, a linear model or the in-

Table 4. Nonlinear multilevel mixed effects model using Berkey-Reed function to represent individual growth; results from the regression of weight (in grams) on age (in weeks), sex and treatment.

Mixed-effects ML non-linear regression						
Fixed-effects Parameters		Coef.	Std. Err.	P>|z|	[95% Conf. Interval]	
a	sex	565.79	33.21	0.000	500.69	630.89
	treatment	-380.53	33.3	0.000	-445.8	-315.25
	_constant	655.28	28.32	0.000	599.77	710.79
b	sex	-0.15	0.12	0.221	-0.39	0.09
	treatment	-0.84	0.19	0.000	-1.23	-0.46
	_constant	1.57	0.15	0.000	1.28	1.86
c	sex	78.15	9.41	0.000	59.7	69.6
	treatment	-118.26	9.44	0.000	-136.77	-99.75
	_constant	89.06	8.01	0.000	73.35	104.77
d	sex	-3381.29	137.82	0.000	-3651.41	-3111.17
	treatment	1330.01	138.13	0.000	1059.28	1600.74
	_constant	-3125.87	117.84	0.000	-3356.83	-2894.92
Random-effects Parameters			Estimate	Std. Err.	[95% Conf. Interval]	
Litter: Identity						
	var(U0)	0.57	0.08		0.43	0.75
	var(Residual)	1966.77	14.56		1938.44	1995.51

Goodness-of-fit M**easures**			
Log-likelihood	-191030	*Df*	14
AIC	382088		
BIC	382207.1		

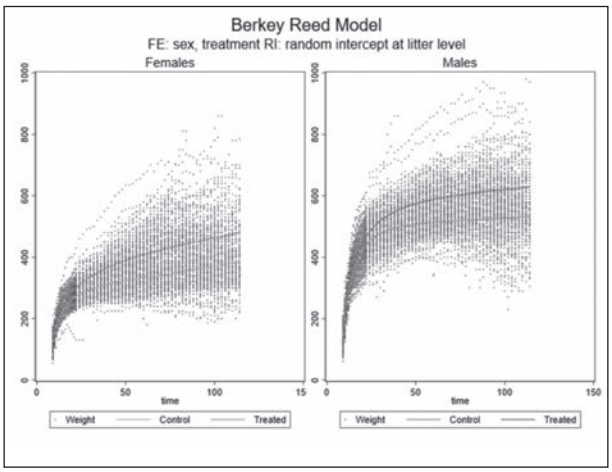

Figure 6. Plot of body weights (in grams) and estimated average predictions, nonlinear multilevel mixed effects model using Berkey-Reed function to represent individual growth

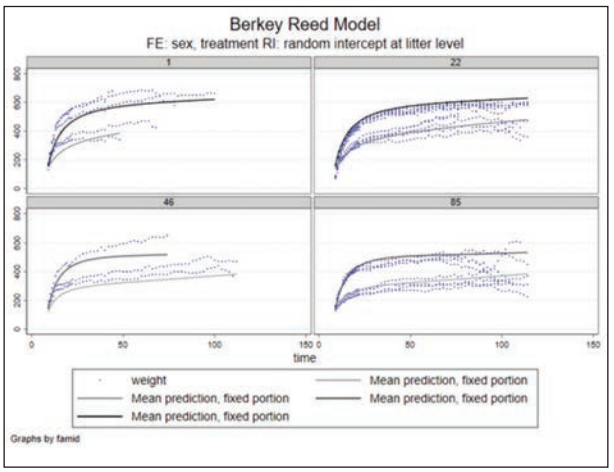

Figure 7. Plot of weights (in gram) and linear predictions with fixed and random part in four randomly selected litters, nonlinear multilevel mixed effects model using Berkey-Reed function to represent individual growth

clusion of a quadratic term to represent time should be the best options, since rarely growth models are built to model the weight trajectories during the whole lifespan of individuals, so a simpler and more efficient alternative is to be preferred.

A possible alternative to build flexible yet simple models are General Additive Models: it could be interesting to evaluate their performance in this context, since they may allow to handle data with such an irregular trend, and at the same time to maintain a simple and understandable interpretation for the regression parameters.

Some issues remain open, like the problem of how to handle the extreme trends that some animals showed in the last part of their life; they represent an interesting feature, that is usually associated with ageing and the onset of pathological conditions (for example mammary tumours increase the individual weight, other tumours decrease individual weight).

Conclusions

Continuous experimental longitudinal data, in particular those consisting in body weights, have some very peculiar characteristics similar to the human counterpart, the most relevant for their analysis are non-linearity and the fact that they can take unexpected, extreme turns upwards or downwards, mostly when rats are close to the end of their life, reflecting the presence of large neoplastic mammary lumps or a worsening of the health conditions due to ageing. These features should discourage the use of methods based on the comparison of measures of synthesis like the group means, because they could be heavily affected by the atypical recordings, giving an unrealistic picture of the situation and possibly preventing to detect subtler differences caused by experimental factors.

The use of multilevel mixed effects models is therefore to be encouraged, since they allow to analyse directly the recordings of each subject, without concerns about the differences in the duration of the follow-up. They are also a precious tool in case of clustered data, like in this rather peculiar experimental design, where no randomization was performed on a whole cohort of rats of second generation. The most straightforward specification of such models using a proper linear function isn't the best option because it requires a transformation of the variables, so the advantage of a simple functional form is counterbalanced by the difficult interpretation of the transformed variables. Even the introduction of polynomial terms, that allow to represent a curve trajectory remaining in the frame of a linear function, slightly reduces the ease of the interpretation, but it can still be acceptable in case it allowed a more faithful representation of the growth trajectories; as these analyses showed, nevertheless, it's not always the case. New tools in this field, that may have potential advantages, are the nonlinear growth models that were "borrowed" from human studies: a wide variety exists, so one can select the most appropriate every time, according to the characteristics of data. The model's parameters are not always easy to interpret, but a clear indication is provided about the direction, magnitude and statistical significance of the effect of each covariate.

As a more general recommendation, deepening the knowledge and understanding of the methods used for the statistical analysis of experimental results is an important strategy to enhance the quality of the research, in particular for toxicology (27). This work is an attempt in this direction: it aims to go beyond the statistical techniques that are routinely used, to explore the characteristics of the data and to try to understand the mechanisms that determined them. In this framework, some methodologies to answer the research questions in a more comprehensive way were proposed and applied to the carcinogenicity bioassay on a sweetened beverage (Coca Cola) performed by the Ramazzini Institute. All estimated models confirm that the unconstrained consumption of Coca-Cola in this experiment was associated with a significant increase in body weight (linear mixed-effect model, logarithmic function of time: $p= 0.021$; linear mixed-effect model, cubic function of time: $p= 0.016$; non-linear mixed-effect model, Berkey-Reed growth function: $p= 0.000$ for all parameters).

To conclude, it's worthy to remind once more the importance of properly choosing the methods and specifying the models, where properly means in a data-and-experience-driven way. A thorough knowledge of the data and of the dynamics that contribute to determine them is always a good starting point to

build plausible, representative and meaningful models. Another crucial point is the fact that model checking and verification of the respect of the assumptions that lie at the foundations of any method, should become a routine embedded in every analysis, while it still remains not so common (or, at least, not always explicitly reported) in the literature regarding carcinogenicity bioassays, in particular for non-cancer outcomes.

Ultimately, the use of more adequate statistical models helps refine and reduce the use of experimental animals, in line with the EU Directive 2010/63/EU (28).

Author's contribution:
Daria Sgargi: concept and design of study, data collection; Daria Sgargi, Simona Panzacchi, Daniele Mandrioli: data interpretation and analysis, drafting, revision, approval of final manuscript.; Rossella Miglio and Fiorella Belpoggi: supervision of data interpretation and analysis, critical revision of the entire text, approval of final manuscript. This work is adapted from the PhD Thesis of Daria Sgargi "The Analysis of Survival and Longitudinal Data from Life-span Carcinogenicity Bioassays on Sprague-Dawley Rats", Department of Statistical Science, University of Bologna.

References

1. Global Burden of Disease Cancer Collaboration. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived with Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015. A Systematic Analysis for the Global Burden of Disease Study. JAMA Oncol. 2017; 3 (4): 524–548
2. International Agency for Research on Cancer. Preamble. In: IARC monographs on the Evaluation of Carcinogenic Risks to Humans. WHO- IARC, Lyon. Last update September 2015
3. Bucher J R. The National Toxicology Program Rodent Bioassay. Designs, Interpretations, and Scientific Contributions. Ann N Y Acad Sci 2002, 982: 198-207
4. Organization for Economic Cooperation and Development. Test No. 451: Carcinogenicity Studies. OECD Publishing, Paris, 2009
5. Organization for Economic Cooperation and Development. Guidance document 116 on the conduct and design of chronic toxicity and carcinogenicity studies, supporting Test Guidelines 451, 452 and 453. OECD Publishing, Paris; 2nd edition, 13 April 2012
6. Shockley K R, Kissling G E. Statistical Guidance for Reviewers of Toxicologic Pathology. Toxicol Pathol 2018, 46(6): 647-652
7. Hothorn L. Statistical evaluation of toxicological bioassays-a review. Toxicol Res, 2014; 3: 418-432
8. Belpoggi F, Tibaldi E, Soffritti M, Falcioni L, Bua L, Trabucco F. Results of long-term carcinogenicity bioassays on Coca-Cola administered to Sprague-Dawley rats. Ann NY Acad Sci 2006; 1076: 736-752
9. Organization for Economic Cooperation and Development. Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies. Paris, OECD, 2002
10. Laird N M, Ware J H. Random effects models for longitudinal data. Biometrics 1982; 38: 963–974
11. Verbeke G, Molenberghs G. Linear Mixed Models for Longitudinal Data. New York Springer, 2000
12. Fitzmaurice G, Molenberghs G. Advances in longitudinal data analysis: an historical perspective. In Davidian M, Verbeke G, Molenberghs G. Fitzmaurice G. Longitudinal data analysis: a Handbook of modern statistical methods, 3-30. London, Chapman & Hall/CRC Press, 2008
13. Pinheiro J, Bates D. Mixed-Effects Models in S and S-PLUS. New York, Springer, 2000
14. West B T, Welch K B, Galecki A T. Linear Mixed Models: A Practical Guide Using Statistical Software, Second Edition. London, Chapman & Hall/CRC Press, 2010
15. Singer J D, Willet J B. Applied longitudinal data analysis. Modeling change and event occurrence. Oxford, Oxford University Press, 2003
16. Lindstrom M, Bates D. Nonlinear mixed-effects models for repeated measures data. Biometrics 1990; 46, 3: 673-668
17. Demidenko E. Mixed Models: Theory and Applications with R, 2nd Edition. Hoboken, Wiley, 2013
18. Hauspie R C, Cameron N, Molinari L. Methods in Human Growth Research. New York, Cambridge University Press, 2004
19. Jenns R M, Bayley N. A mathematical method of studying the growth of a child. Hum Biol 1937, 9: 556-563
20. Count E. Growth pattern of the human physique. Hum. Biol. 1943, 15: 1–32
21. Berkey CS, Reed RB. A model for describing normal and abnormal growth in early childhood. Hum Biol. 1987 Dec; 59 (6): 973-87
22. Sengupta P. The laboratory rat: Relating its age with human's. Int J Prev Med 2013; 4: 624-30
23. Soffritti M, Belpoggi F, Degli Esposti D. Cancer Prevention: the lesson from the lab. In: Biasco G, Tanneberger S. Cancer Medicine at the Dawn of the 21st Century: the view from Bologna 49-64. Bologna, Bonomia University, 2006
24. Calle E E, Rodriguez C, Walker-Thurmond K, Thun M J. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. Adults. N Engl J Med, 2003: 1625-38
25. Rapp K, Schroeder J, Klenk J, Stoehr S, Ulmer H, Concin H, Diem G, Oberaigner W, Weiland SK. Obesity and incidence of cancer: a large cohort study of over 145000 adults in Austria. Br. J. Cancer 2005; 93: 1062–1106
26. Lauby-Secretan B, Scoccianti C, Loomis D, Grosse Y, Bianchini F, Straif K. Body fatness and cancer – viewpoint of

the IARC Working Group. N Engl J Med, 2016; 375:794-798
27. Mandrioli D, Silbergeld E K. Evidence from Toxicology: The Most Essential Science for Prevention. Environ Health Perspect. 2016 Jan; 124 (1): 6–11
28. Eurpean Parliament and Council. Directive 2010/63/UE of Eurpean Parliament and Council, of September 22nd, 2010, on the protection of animals used for scientific purposes (Text with EEA relevance) OJ L 276, 20.10.2010, p. 33–79

Correspondence:
Fiorella Belpoggi,
Cesare Maltoni Cancer Research Center, Ramazzini Institute
Via Saliceto, 3, 40010 Bentivoglio, Bologna, Italy
Tel. +39 051 6640460
Fax +39 051 6640223
E-mail: belpoggif@ramazzini.it

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 99-102, 2018 © Mattioli 1885

The problems of rendering psychological assistance to oncological patients in the region of Russian Federation

Leskina Eleonora Igorevna
Saratov State Law Academy, Russian

Summary. Among the most urgent problems of our time, oncological diseases occupy a special place. When the diagnosis sounds like a sentence, when people need highly qualified and expensive help, the state can not stay away. And one of the types of social security here is qualified medical psychological assistance. *The aim of the article:* Is to study various aspects of psychological care for cancer patients in the Saratov region. *Methodological basis:* The methods used are both empirical (analysis and synthesis, induction and deduction, systematization), and theoretical (methods of constructing and researching the object of research and methods of constructing and justifying theoretical knowledge) levels. *Results:* The dynamics and prevalence of people who need psychological help in connection with the diagnosis of cancer is shown. The article considers the history of medical care for cancer patients in the Saratov region, the main forms of providing psychological assistance to cancer-patients in the region. *Conclusions:* Currently, there is a shortage of cancer-psychologists in the region, it is necessary to provide psychological support for cancer -psychologists to persons who underwent preventive operations in connection with the high probability of hereditary cancer within the framework of MHI.

Key words: oncological patients, cancer, psychological help, Russian Federation, Hospice, Saratov region, oncopsychologists, support, social security

President of the Russian Federation V.V. Putin in the annual Address as one of the priority areas of state policy called the fight against oncological diseases. Indeed, in Russia, almost everyone faced oncology among their friends or relatives. Particularly interesting is the state of the problem and the activities carried out at the regional level.

Currently, the Saratov region is the 29th in the Russian Federation and the 7th in the Volga Federal District by the number of cancer patients. In 2017, according to the chief oncologist of the region Vladimir Semenchenya, for the first time cancer was detected in 11 thousand people, which is 300 more people than last year. Statistics of the dynamics of life expectancy in Russia do testify to the growth of these indicators, but these data are not proportional to the growth of cancer among the population. It should be noted that the number of detected oncological diseases is increasing in other regions (1).

I would like to draw special attention to the provision of psychological assistance on the basis of the Regional Oncology Dispensary.

According to Art. 4 FZ N 323-FZ "On the fundamentals of protecting the health of citizens in the Russian Federation", the priority of the patient's interests in the provision of medical care is the most important principle for ensuring the protection of public health. A medical psychologist is a subject whose activities are necessary in the field of oncology. From the quality of the provision of psychological assistance, the

qualification of a medical psychologist, many factors depend: the course of the disease, the condition of the patient and his family members, etc. (2).

In general, psychological care in oncology is one of the most important types of social security for cancer patients and their relatives. Accompanied by the oncologist, in one form or another, 90% of patients and up to 40% of those close to them need. The defining thing here is that the very diagnosis of «cancer» for both patients and their family members often sounds like a sentence. Typical in these cases, the suddenness of the detection of the disease, a dramatically changed situation: he was healthy, became deadly sick. This causes a feeling of confusion, impasse, devaluation of the old life experience.

The importance of medical psychological assistance to cancer patients and their relatives is noted at the international level. June 7, 2016 in Bishkek adopted a decision of the Council of Heads of Government of the CIS «On the Concept of Cooperation of the Member States of the Commonwealth of Independent States in the field of counteracting cancer». One of the main areas of cooperation in the field of prevention, early diagnosis and treatment of oncological diseases is cooperation in the training, retraining and professional development of specialists in the field of medical psychology. Also, among the main goals, the formation of a relation to oncological diseases as curable diseases among the population, as well as ensuring the principles of continuity in the implementation of medical, psychological and social rehabilitation of patients with oncological diseases is noted.

Among the often diagnosed disorders, anxious-phobic, panic and depressive conditions, as well as neurasthenia and post-traumatic stress disorders, are revealed in patients.

The principle of priority of the patient's interests is to provide medical care to the patient, taking into account the patient's desire, his physical condition, while observing the cultural and religious traditions of the patient, if possible. And this becomes especially urgent when providing psychological assistance to cancer patients (3).

In the Saratov region, the psychological support of cancer patients and their families began to develop recently. Only in 2015, on the basis of the Regional Oncology Dispensary, a psychological service was established. At the same time, the number of people seeking psychological help is only increasing. So, in 2017, in comparison with the previous year, the number of consultations of a psychologist doubled, amounting to 485 consultations. As of April 2018, there are three oncopsychologists working in the Saratov region. At first glance, this is not much. However, considering that the number of oncopsychologists in the country is 210 people, the region's indicators look quite optimistic.

Such an increase in the need for psychological care specialists explain not only with the increase in the number of cancer patients. There are two main factors that determine the increased and increasing need for psychological assistance:

1) recognition by doctors of an interdisciplinary approach in the treatment of cancer. Attention not only to the physical, but also the psychological state of the patient helps to activate the inner potential of the person and increase the chances of improving and stabilizing the process;

2) people's understanding that the diagnosis of oncological pathology and the diagnosis is a serious psychogeny for the patients themselves and for their families. Oncological disease consists of a series of psychotraumatic events occurring at different phases of the disease, so recourse to psychological help is a more natural process in practice.

Currently in the Saratov region, psychological assistance is provided in the following forms:

1) the medical psychologist provides psychological assistance to oncological patients and their relatives (with their written consent) in inpatient and outpatient settings according to the direction of the doctor of the polyclinic or hospital or by the method of selection by the psychologist himself in the departments;

2) psychological rehabilitation assistance to patients is provided on the basis of the interaction of doctors - oncologists, radiologist doctors, surgeons, who refer patients to a consultation with a medical psychologist. Psychological rehabilitation is provided using modern methods of psychodiagnostics, psychological counseling and psychological correction and non-drug therapy.

3) daily (on weekdays) reception of patients in the office of a medical psychologist is carried out. For each

patient a psychological examination card is drawn up, indicating the results of the conducted psychodiagnostics and recommendations. For the patients of the dispensary, a hot-line telephone is available on weekdays on a weekly basis to contact a psychologist.

An important point is the rendering of a psychologist specifically non-pharmacological assistance, since the patient's body is already weakened by the influence of drugs and methods of treatment (chemotherapy, radiation therapy, etc.).

A special place in the work of a medical psychologist is occupied by corrective psychotherapy in the departments of the dispensary's hospital. At the preoperative stage, individual therapy of fear of surgical treatment and anesthesia is carried out with patients using short-term (focus) psychotherapy, rational psychotherapy and trance techniques.

In the postoperative period, individual and group work is carried out using different directions of art therapy, rational and positive and relaxation psychotherapy. Diagnostics and individual correction of attitude to illness and health are necessarily carried out, when non-rational types are identified.

Also in the work with patients, the Simontonov program is applied. This psychotherapeutic program is the most effective, known and recognized in oncopsychology. A method based on visualization of the process of recovery, self-suggestion and introspection.

The goal of the psycho-correctional work of the psychologist is aimed at harmonizing the emotional state of the individual, improving the quality of life of the patient, the social environment (relationships with family and significant people), with the aim of both reducing and preventing social restrictions caused by the oncological disease.

It should be said about the «School of the patient» in the Saratov regional oncological dispensary, where, in particular, the psychologist organizes various classes on various issues of psychological rehabilitation of cancer patients. Psychologists and doctors of the Saratov regional oncology dispensary noted improvement in the psychological state, quality of life and well-being of patients using methods of psychotherapy in their course of treatment.

Psychologists of the oncological dispensary constantly improve their knowledge, participate in the annual congress of oncopsychologists of Russia in Moscow.

We note the shortcomings of the legal regulation of social security in the form of psychological assistance to oncological patients. First of all, the interconnection of certain hereditary factors of cancer development has been discovered in the world practice (4). Accordingly, the effectiveness of planned operations for the removal of suspected dangerous organs and tissues has been proven. So, in the world oncology practice a good effect of preventive operations is shown: for the prevention of ovarian cancer - bilateral salpingo-ovariectomy, which reduces morbidity and mortality from breast and ovarian cancer. Such an operation is shown to carriers of mutations in the BRCA1 or BRCA2 genes at the end of the reproductive period (the optimal age is 35 to 40 years) (5). However, these patients are not recognized as cancer patients, respectively, in addition to the fact that these operations can only be carried out on a fee basis because they are not included in the Program of State Guarantees of Free Medical Assistance to Citizens. These patients are deprived of the help of oncopsychologists within the framework of CHI. At the same time, these planned operations injure the patient's psyche, these patients need qualified help of narrow-minded medical psychologists.

Another problem is the lack of narrow specialists. Oncopsychologist for today a rare specialty. Most psychological methods of supporting patients in general are fairly well known. In the Saratov region, currently there is a shortage of these specialists, and this despite the availability of a medical university in the region.

Among the shortcomings of the provision of psychological assistance in the Saratov region, one can note the absence of a hospice. These institutions provide specialized psychological and spiritual help not only to incurable patients of oncological, but also therapeutic, neurological, pediatric profile with the aim of providing them with palliative care, psychosocial rehabilitation, and psychological and social support for relatives for the period of close and loss. So, the hospice in the Volgograd region, even at the level of the visiting brigade's inspection, includes a medical psychologist in this brigade without fail (6).

The complexity of the device of the psychological service is that, according to the OMS, the patient in

our country can receive only what is prescribed in the standard of medical care. For any disease there is such a standard, developed and signed by the Ministry of Health. But in the standards for oncology there is no such kind of services as «accompaniment of psychologist-psychotherapist».

To summarize, we note that in general, the experience of the Saratov region in providing psychological assistance to cancer patients and their relatives could be used in other regions.

References

1. Sidorov SV, Krasilnikov SE, Babayants EV, Chernus N.Yu. The patient's right to choose the method of medical care in the treatment of cancer. Medical Law 2017; 4: 23-7.
2. Krasilnikov S E, Babayants E K, Chernus NYu. Legal aspects of the implementation of preventive oophorectomy in oncological health care institutions. Medical Law 2017; 5: 42-5.
3. Sidorov SV, Krasilnikov SE, Babayants EV, Chernus N.Yu. The patient's right to choose the method of medical care in the treatment of cancer. Medical Law 2017; 4: 23-7.
4. Lazarev AF, Zadontseva NS, Gofman AA Hereditary cancer of the breast. Russian Cancer Journal 2014; 2: 40-5.
5. Lyubchenko LN, Bateneva EI, Abramov IS, Emelyanova MA et al. Hereditary cancer of the breast and ovaries. Malignant tumors 2013; 2: 53-61.
6. Ertel LA, Porokh LI Organizational and legal aspects of rendering socio-medical assistance to patients with cancer: problems and prospects. Social and pension law 2015; 3: 33-8.

Correspondence:
Leskina Eleonora Igorevna
Saratov state law academy, Russian
E-mail: elli-m@mail.ru

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 103-108, 2018 © Mattioli 1885

rs3798577 polymorphism located in a putative miRNAs target site of estrogen receptor 1 reduced breast cancer risk in an Iranian population

Nafiseh Reisi Dehkordi[1], Soha Parsafar[2], Kamran Ghaedi[3†], Maryam Peymani[1]

[1] Department of Biology, Faculty of Basic Science, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran; [2] Department of Industrial and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran; [3] Cellular and Molecular Biology Division, Biology Department, Faculty of Sciences, University of Isfahan, Isfahan, Iran

Summary. *Purpose:* In the current case-control study, the possible association between rs3798577, a microRNA-related SNP located on *ESR1* 3'-untranslated regions (3'-UTR), and breast cancer was evaluated in Iranian women for the first time. *Materials and Methods:* 126 breast cancer patients and 141 hospital healthy controls were enrolled in this study. Genotyping of the selected SNP in *ESR1* was disclosed using the allele-specific primer polymerase chain reaction (ASP-PCR) assay. Odds ratio, 95% confidence interval, and p value were calculated to examine the association between SNP and breast cancer related clinical features. In addition, an in silico prediction was performed to identify potential functionality of the SNP within miRNA binding sites in the 3'-UTR of *ESR1*. *Results:* The T allele carriers of the SNP had significantly inverse association with BC incidence (T/T and C/T vs C/C; OR, 0.50; 95% C.I., 0.27-0.92; P value, 0.025). In addition, T allele carriers conferring decreased risk of metastasis, ER/PR negativity, HER2 positivity, and stage IV incidence and also increased risk of BC death and grade III incidences but these results did not reach statistical significance. Bioinformatically, rs3798577 is located on *ESR1* 3'-UTR within the potential target sequence of miR-1278 and miR-125b-2-3p. Hence, the T allele may increase miRNA-mRNA binding strength. *Conclusion:* The results showed that the *ESR1* rs3798577 T allele significantly reduced breast cancer risk, in agreement with bioinformatical results. The association between rs3798577 genotypes and breast cancer has been reported contradictorily in different studies. Furthermore, the bioinformatical results need to verify. Therefore, further studies with large sample size and functional assessment are strongly suggested.

Key words: breast cancer, *ESR1*, functional SNP, 3'-UTR

1. Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy among women with heterogeneous clinical, genetic, and biochemical features (1, 2). Estrogen induces proliferation of mammary epithelial tissue by interacting with nuclear receptors, estrogen receptors estrogen receptors (ER α and β), acting as a transcription factor and can be used for therapeutic purposes (2). ER α expression has been used for predicting responsiveness to hormone therapy, and loss of its expression is associated with poorer BC outcomes (3, 4). As previous studies have shown (5, 6), *ESR1*, encodes estrogen receptor α, is a strong candidate gene for BC association studies.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that bind to 3'-untranslated regions (3'-UTRs) of target mRNAs and mediate trans-

lational inhibition or cleavage; thus, it may participate in various pathological events (7, 8). Many studies have aimed to characterize functional single nucleotide polymorphisms (F-SNPs) related to miRNA regulation process. These variants are categorized into the two main groups. Firstly, precursor miRNAs (pre-miRNAs) polymorphisms may cause miRNA aberrant expression possibly via altering pre-miRNA stability. Secondly, miRNA target sites (3'-UTR of targets) polymorphisms may modify miRNA-mRNA binding strength. Bioinformatics tools are useful to predict the effects of SNPs at miRNA loci and targets and offer possible descriptions for the phenotype associations (9, 10).

Here, we hypothesized that the *ESR1* 3'-UTR SNP, rs3798577 (c.*1029C>T) genetic variation can alter the expression of *ESR1* and its downstream signaling through miRNA interactions; hence, may affect BC susceptibility. Briefly, we genotyped the rs3798577 SNP in a cohort of Iranian BC patients in order to search for associations between the polymorphism and BC and its clinicopathological characteristics, including stage, grade, early metastasis status, hormone receptors (ER and PR), and HER2/neu overexpression. In addition, an in silico assessment was performed to investigate possible function of the selected SNP.

2. Materials and methods

2.1 Ethics Statement

Written informed consent for the genetic study was obtained from all participants. This study was approved by the institutional review board of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

2.2 Patients and healthy controls

Genomic DNA was isolated from blood samples of 126 unrelated Iranian patients with BC and 141 women without family history of any type of cancers using the PrimePrep Genomic DNA Isolation Kit (GeNetBio, Chungnam, South Korea) according to the manufacturer's protocol.

2.3 Pathological diagnosis and grading

Clinicopathological characteristics and follow-up data were obtained from the files of reference pathology Laboratories where immunohistochemistry (IHC) and pathological tests are performed centrally by experienced operators and a dedicated pathologist who tracks strict sample handling, processing and reporting protocols, thus ensuring the reliability of results. The pathological and clinical attributes of the patients are listed in Table 1.

2.4 SNP genotyping

Allele-specific primer polymerase chain reaction (ASP-PCR) assay was applied for SNP genotyping (11). SNP genotyping was performed by using the C allele specific forward (5'GGC ATG GAG CTG AAC AGT AAC3') T allele specific forward (5'GGC ATG GAG CTG AAC AGT AAT3'), and common reverse (5'AAT GAA GAA GAG CTG GAC TAC CC3') primers. Standard cycling was performed in a thermocycler (ASTEC PC-818; ASTEC, Fukuoka, Japan) under the following conditions: Initial denaturation at 94°C for 5 min followed by 33 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 40 sec, and finally 72°C for 7 min. PCR reaction with C allele specific forward and T allele specific forward primers are performed separately (two reactions are needed for each sample). The allele specific PCR product was electrophoresed by 1.5% agarose gel electrophoresis in 1X

Table 1. The clinicopathologic characteristics of the patients with breast carcinoma

Characteristics	Number
Histological grade	I: 12, II: 48, III: 36, Unknown: 30
Stage	I: 18, II: 15, III: 15, IV: 66, Unknown: 12
Estrogen receptor (ER) status	Positive: 63, Negative: 18, Unknown: 45
Progesterone receptor (PR) status	Positive: 57, Negative: 24, Unknown: 45
HER2 status	Positive: 24, Negative: 57, Unknown: 45

Tris-Borate-EDTA buffer at 100 V and stained with RedSafe Nucleic Acid Staining solution (Boca Scientific, Inc., Boca Raton, FL, USA) for visualization. The amplicon sizes for *ESR1* 3'-UTR variant (rs3798577) was 121 bp for both alleles (Figure 1).

2.5 Bioinformatic analysis

miRNASNP version 2.0 (12) was used to predict impact of the rs3798577 *ESR1* 3'-UTR SNP based on miRNA binding free energy comparison of the two SNP alleles. To illustrate, this database was utilized to computationally estimate the impact of rs3798577 in modifying the affinity between miRNAs and 3'-UTR of *ESR1* mRNAs (either gain or loss) based on alterations in Gibbs free energy of binding reaction.

2.6 Statistical analysis

Deviation from Hardy-Weinberg equilibrium (HWE), odds ratios (ORs) with 95% confidence intervals (CIs), and Armitage's trend test were achieved using DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Noticeably, Armitage's trend test performs association assessment with considering the individuals' genotypes rather than just the alleles for and also without relying on the assumption of HWE, following the guidelines provided by Sasieni (13).

Consistency with HWE was examined by the exact test. In addition, association tests were evaluated using chi-square test. Logistic regression models were used to account odds ratios (OR) and related 95% confidence intervals (95% CI). P-value of <0.05 was considered statistically significant.

3. Results

*3.1 Frequencies of ESR1 3'-UTR variant rs3798577 (c.*1029C>T)*

Among all individuals, including patients and healthy controls, alleles and genotypes were distributed as following frequencies: C, 0.63; T, 0.37; C/C,

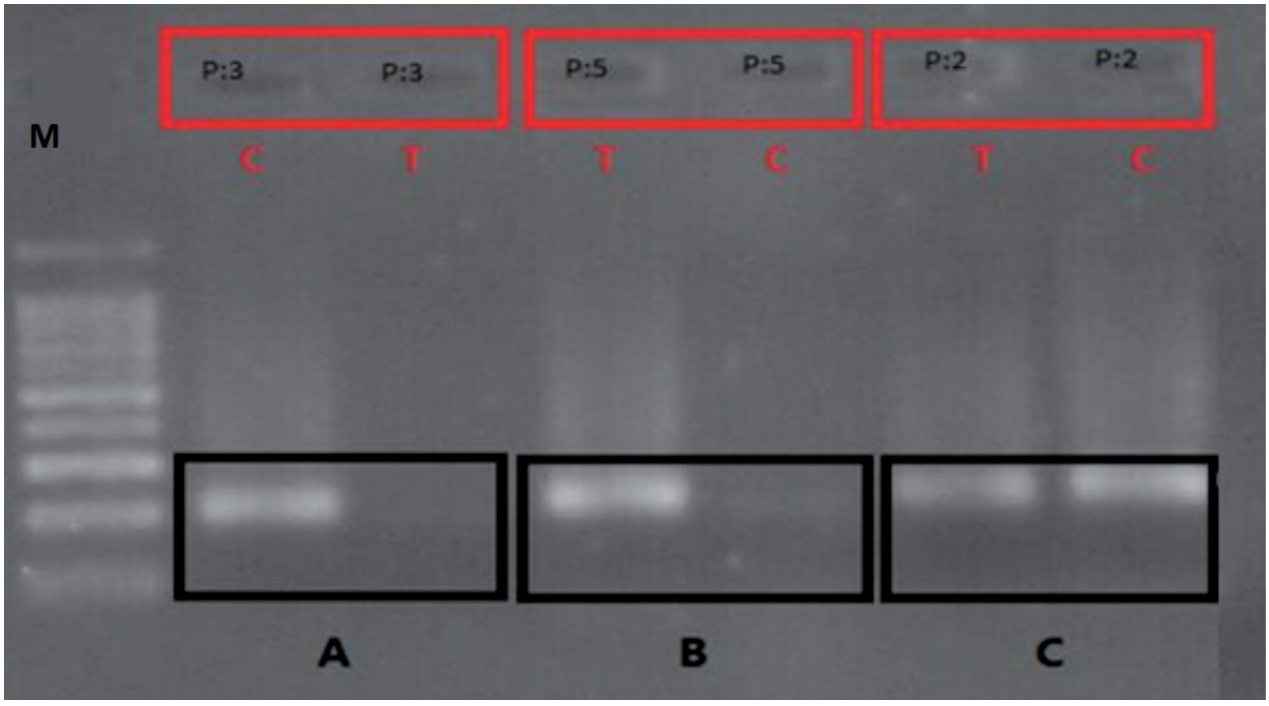

Figure 1. Representative PCR amplicons of allele-specific primer PCR resolved by 1.5% agarose gel electrophoresis to detect the ESR1 3'-UTR variant rs3798577 polymorphism genotyping. Lanes 1 and 2: C/C; lanes 3 and 4: T/T; lanes 5 and 6: C/T; M: DNA marker

0.41; C/T, 0.44; T/T, 0.15. The observed genotype frequencies showed that rs3798577 SNP was in HWE proportions in the population of the study (P value, 0.517).

3.2 Associations with BC and tumor phenotypes

To test the relationships between the SNP and prognostic tumor phenotypes, allele frequency differences, Armitage's trend test, and genotypic association (assuming a dominant model of inheritance for T allele) were performed by Pearson's chi test and OR calculation (Table 2). From the all association tests with various events, T allele carriers (T/T and C/T) were inversely associated with BC compared with C/C genotype (OR, 0.50; 95% C.I., 0.27-0.92; P value, 0.025). In addition, T allele carriers conferring decreased risk of metastasis, ER/PR negativity, HER2 positivity, and stage IV incidence and also increased risk of BC death and grade III incidences but these did not reach statistical significance (P value >0.05).

3.3 Bioinformatical results

Computational predictions proposed that rs3798577 is located on *ESR1* 3'-UTR within the potential target sequence of miR-1278 and miR-125b-2-3p. As a result, the T allele may increase miRNA-mRNA binding occurrence (Table 3).

4. Discussion

Associations between variants in *ESR1* and BC risk have evaluated by previous studies (6, 14) and were extensively reviewed by Herynk et al. (15). Estrogen induced ERα can directly bind to estrogen response elements or indirectly interacts with chromatin via binding to other transcription factors, such as coactivators or corepressors (16). Dysregulation of ERα can affect BC progression and susceptibility (17).

Rs3798577 is located on 3'-UTR of *ESR1* and predicted target sequence of miR-1278 and miR-

Table 2. Allelic, Armitage's trend, and genotypic association tests between rs3798577 and characteristics of patients

Variable comparison	Allelic association (T *vs* C)		Armitage's trend test (T *vs* C)		Genotypic association (T/T + C/T *vs* C/C)	
	OR (95% CI)	*P*	OR	*P*	OR (95% CI)	*P*
BC vs Control	0.81 (0.52-1.26)	0.357	0.91	0.373	**0.50 (0.27-0.92)**	**0.025**
Metastatic BC vs Non-metastatic BC	0.62 (0.31-1.24)	0.172	0.70	0.241	0.62 (0.25-1.53)	0.297
BC death vs Survival	1.64 (0.71-3.75)	0.241	1.45	0.315	1.58 (0.50-5.04)	0.434
ER- vs ER+	0.74 (0.26-2.09)	0.573	0.87	0.620	0.55 (0.14-2.11)	0.380
PR- vs PR+	0.72 (0.28-1.84)	0.494	0.79	0.547	0.67 (0.20-2.20)	0.505
HER2+ vs HER2-	0.72 (0.28-1.84)	0.494	0.79	0.547	0.67 (0.20-2.20)	0.505
Stage IV vs Stage I/II/III	0.61 (031-1.22)	0.162	0.65	0.224	0.78 (0.32-1.92)	0.587
Grade III vs Grade I/II	1.72 (0.78-3.82)	0.179	1.50	0.235	1.86 (0.66-5.21)	0.237

Table 3. An in silico analysis of the SNP-miRNA binding

miR	miR sequence	miR site on ESR1 3'-UTR with rs3798577	Effect (T allele *vs* C allele)
miR-1278	UAGUACUGUGCAUAUCAUCUAU	AUGGAGCUGAACAGUAC[C/U]	Gain
miR-125b-2-3p	UCACAAGUCAGGCUCUUGGGAC	CUGAACAGUAC[C/U]UGUG	Gain

125b-2-3p. Therefore, in the present study, *ESR1* 3'-UTR SNP, rs3798577 (c.*1029C>T) was selected and genotyped in an Iranian population comprising 126 BC cases and 141 cancer-free controls. Although, the C allele was introduced as a minor allele in the rs3798577 location in various populations at dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), we observed lower frequency for the C allele compare with the T allele in the Iranian population. In our cases, we found inverse significant association between the T allele carriers and BC incidence (OR, 0.50; 95% C.I., 0.27-0.92; P value, 0.025). In accordance with our results, the C allele was strongly associated with the risk of BC in Caucasian population (18). Possible functional mechanism of this SNP bioinformatically attributed to changes in ERα expression by enhancing the target sequence of miR-1278 and miR-125b-2-3p due to C>T substitution. In addition, the C allele has been reported that to be associated with surviaval and distant metastasis (18, 19) which is in agreement with our nonsignificant observations (Table 2). Moreover, additional associations between the SNP and other BC characteristics were not statistical significant in the present study.

Inconsistent with our finding, this SNP was not statistically associated with BC risk in other studies (6, 14, 20). In addition, converse with our and others observation, the T allele frequency of rs3798577 was significantly higher in BC cases than controls in Chinese and Korean populations (21, 22). This supports the fact that associations between polymorphisms and complex phenotypes can be varied mostly due to ethnicity background and variant allele frequencies of polymorphisms; hence, association results should not be generalized between different populations.

To our knowledge, this is the first investigation which was aimed to evaluate the possible association between rs3798577 and BC phenotypes among Iranian women. This case–control study showed an inverse association between the T allele carriers and BC incidence (T/T and C/T are protective genotypes) which is interestingly consistent with the bioinformatical results. It can be concluded that the T allele may strengthen *ESR1* mRNA-miRNA binding; thus, it may lead to *ESR1* downregulation and better BC outcomes. However, limitations of the current study, including the small sample size and unvalidated bioinformatics results need to ameliorate in further investigations. All in all, the rs3798577 polymorphism functionality in BC is still inconclusive and more examinations are required to test the association of this polymorphism in BC and also its biological importance.

5. Compliance with Ethical Standards

Written informed consent for the genetic study was obtained from all participants and also this study was approved by the institutional review board of Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA: a cancer journal for clinicians 2015; 65(1): 5-29.
2. Sommer S, Fuqua SA, editors. Estrogen receptor and breast cancer. Seminars in cancer biology; 2001: Elsevier.
3. Ellmann S, Sticht H, Thiel F, Beckmann MW, Strick R, Strissel PL. Estrogen and progesterone receptors: from molecular structures to clinical targets. Cellular and molecular life sciences 2009; 66(15): 2405-26.
4. Jensen EV, Jordan VC. The estrogen receptor a model for molecular medicine. Clinical Cancer Research 2003; 9(6): 1980-9.
5. Kallel I, Rebai M, Rebai A. Mutations and polymorphisms of estrogens receptors genes and diseases susceptibility. Journal of Receptors and Signal Transduction 2012; 32(6): 304-13.
6. Li N, Dong J, Hu Z, Shen H, Dai M. Potentially functional polymorphisms in *ESR1* and breast cancer risk: a meta-analysis. Breast cancer research and treatment 2010; 121(1): 177-84.
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116(2): 281-97.
8. Mesrian Tanha H, Mojtabavi Naeini M, Rahgozar S, Moafi A, Honardoost MA. Integrative computational in-depth analysis of dysregulated miRNA-mRNA interactions in drug-resistant pediatric acute lymphoblastic leukemia cells: an attempt to obtain new potential gene-miRNA pathways involved in response to treatment. Tumor Biology 2015: PubMed ahead of print.
9. Jin Y, Lee CG. Single nucleotide polymorphisms associated with microRNA regulation. Biomolecules 2013; 3(2): 287-302.
10. Bagheri F, Mesrian Tanha H, Mojtabavi Naeini M, Ghaedi K, Azadeh M. Tumor-promoting function of single nu-

cleotide polymorphism rs1836724 (C3388T) alters multiple potential legitimate microRNA binding sites at the 3'-untranslated region of ErbB4 in breast cancer. Molecular Medicine Reports 2016; 13(5): 4494-8.
11. Mesrian Tanha H, Mojtabavi Naeini M, Rahgozar S, Rasa SMM, Vallian S. Modified Tetra-Primer ARMS PCR as a Single-Nucleotide Polymorphism Genotyping Tool. Genetic testing and molecular biomarkers 2015; 19(3): 156-61.
12. Gong J, Liu C, Liu W, Wu Y, Ma Z, Chen H, et al. An update of miRNASNP database for better SNP selection by GWAS data, miRNA expression and online tools. Database 2015; 2015: bav029.
13. Sasieni PD. From genotypes to genes: doubling the sample size. Biometrics 1997: 1253-61.
14. Wang Y, He Y, Qin Z, Jiang Y, Jin G, Ma H, et al. Evaluation of functional genetic variants at 6q25. 1 and risk of breast cancer in a Chinese population. Breast Cancer Res 2014; 16(4): 422.
15. Herynk MH, Fuqua SA. Estrogen receptor mutations in human disease. Endocrine reviews 2004; 25(6): 869-98.
16. Thomas C, Gustafsson J-Å. The different roles of ER subtypes in cancer biology and therapy. Nature Reviews Cancer 2011; 11(8): 597-608.
17. Holst F, Stahl PR, Ruiz C, Hellwinkel O, Jehan Z, Wendland M, et al. Estrogen receptor alpha (*ESR1*) gene amplification is frequent in breast cancer. Nature genetics 2007; 39(5): 655-60.
18. Anghel A, Raica M, Narita D, Seclaman E, Nicola T, Ursoniu S, et al. Estrogen receptor alpha polymorphisms: correlation with clinicopathological parameters in breast cancer. Neoplasma 2010; 57(4): 306-15.
19. Tapper W, Hammond V, Gerty S, Ennis S, Simmonds P, Collins A, et al. The influence of genetic variation in 30 selected genes on the clinical characteristics of early onset breast cancer. Breast Cancer Research 2008; 10(6): 1-10.
20. Einarsdóttir K, Darabi H, Li Y, Low LY, Li YQ, Bonnard C, et al. *ESR1* and EGF genetic variation in relation to breast cancer risk and survival. Breast cancer research 2008; 10(1): 1-9.
21. Zhang L, Gu L, Qian B, Hao X, Zhang W, Wei Q, et al. Association of genetic polymorphisms of ER-α and the estradiol-synthesizing enzyme genes CYP17 and CYP19 with breast cancer risk in Chinese women. Breast cancer research and treatment. 2009; 114(2): 327-38.
22. Son BH, Kim MK, Yun YM, Kim HJ, Yu JH, Ko BS, et al. Genetic polymorphism of *ESR1* rs2881766 increases breast cancer risk in Korean women. Journal of cancer research and clinical oncology 2015; 141(4): 633-45.

Correspondence:
Kamran Ghaedi
Professor of Molecular and Cellular Genetics,
Division of Cellular and Molecular Biology,
Department of Biology, Faculty of Sciences,
University of Isfahan,
Postal Code: 81746-73441, Isfahan, Iran
Tel. 0098-31-37932479
Fax 0098-31-37932456
E-mail: kamranghaedi@yahoo.com; kamranghaedi@sci.ui.ac.ir

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 109-111, 2018 © Mattioli 1885

Rare case of hypodiagnostics of subungual melanoma complicated by paraungual panaritium

Evgeny Yurievich Neretin

Doctoral Student of N.N. Blokhin Russian Cancer Research Center, Cancer specialist of the Consultation Department, Samara Regional Clinical Oncological Dispensary

Summary. Subungual melanoma in coexistence with the purulent inflammatory process occurs rather rarely in clinical practice. In spite of its outer localization and accessibility of examination patients are diagnosed mainly with III-IV stages by primary care physicians. We report our experience with clinical status of a 56 year old man that was inaccurately diagnosed with paraungual panaritium that coexisted with the purulent inflammatory process that led to the wrong surgical treatment. The presented case report shows that the doctor may reach an accurate diagnosis earlier with the use of stepwise algorithmic methods for diagnosing malignancy. Reaching the right diagnosis can be achieved taking into consideration both clinical and dermatoscopic clues.

Key words: subungual melanoma, acral melanoma, skin melanoma

Introduction

One of the main problems of early diagnosis of skin melanoma is timely case detection. Skin melanoma morbidity is increasing rapidly in the whole world while skin melanoma mortality rate is not decreasing considerably. At the same time the frequency of cutaneous melanoma and skin cancers occurrence ranges widely in different countries (1). On the whole, there is a widespread growth of morbidity and mortality rate (2). Throughout the world cutaneous melanoma has the highest mortality rate of all skin neoplasms, and in most cases its treatment starts with the distribution of tumor outside the dermis (3). The fundamental methods of making a diagnosis is the clinical method. It includes skin examination, questioning, asking about complaints and anamnesis with the evaluation of tumor according to numerous well-known mnemonic algorithms (the ABCD rule, the EFG rule). But it isn't characterized by a high degree of accuracy (about 37%) when it is used by primary care physicians. As a result in 32,5% of cases cutaneous melanoma is detected at III-IV stages. It worsens the prognosis of the disease. But when primary care physicians study criteria and algorithms of malignant neoplasms the accuracy of diagnostics increases considerably and ranges from 76,2 to 84 % (4-7).

Case report

In 2011 a 56 year old Russian man came to the Oncological centre because of ulcer on the surface of the tumor, color change at the basis of nail plate, pain in the area of the 1st finger on the left hand. While history taking, the patient related that the day before he had had a trauma. He hammered a nail unsuccessfully and he accidentally hit the finger with a hammer. Then he had a pain. Three days later, he came to the doctor. The patient couldn't say whether there had been pigmentation at the basis of nail plate before he consulted the doctor. The patient had skin phototype 2. On physical examination his skin was of ordinary color, peripheral lymphatic nodes were not enlarged. Other organs and systems were without evidence of any pathology.

Additional instrumental examinations such as ultrasonic examination of the abdominal organs and peripheral lymph nodes, X-ray examination of the chest organs were not conducted. On visual examination the surgeon made a preliminary diagnosis of paraungual panaritium. The surgical treatment was provided – dissection and drainage of the purulent cavity. Pigmentation and ulcer on the surface of the tumor at the basis of nail plate were considered to be manifestations of the inflammatory process. The patient was provided with out-patient care. He went for the dressing. The course of oral antibiotics therapy was administered. As a result of the complex treatment, no evidence of recurrence was found. Three months later, the localized recurrence in the area of the middle phalanx was detected. Pigmented lesions at the basis of nail plate and ulcer on the surface of the tumor were preserved. The patient complained about the increased lymph nodes in the left axillary area. The malignant process was suspected and the patient was referred to the Samara Regional Oncological Dispensary for further evaluation.

In the Dispensary a complex research was conducted according to ASCO standards which included examination (figure 1), digital dermatoscopy without immersion (figure 2) and doing sampling of biological material with the subsequent histological investigation and the diagnosis of skin melanoma was confirmed. On ultrasound examination of regional lymph nodes evidence of the metastatise process was discovered in the left axillary lymph nodes. Then the patient was given a combined surgical treatment which included

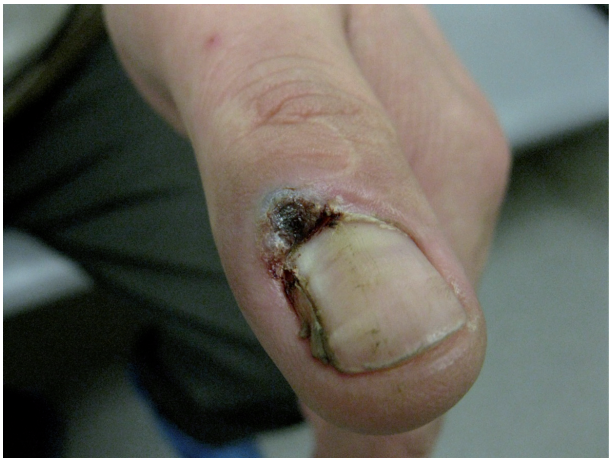

Figure 1. Visual examination

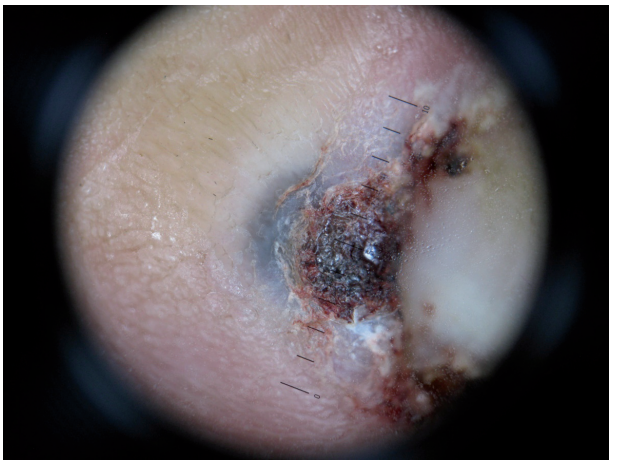

Figure 2. Digital dermatoscopy without immersion

amputation exarticulation of the thumb 1st finger of the left hand and dissection of the left axillary lymph node. On histological examination of the amputation specimen and a block of peripheral lymph nodes the diagnosis of skin melanoma was confirmed. Immunotherapy was administered. The patient was treated with Interferon alpha in a dose of 3 million IU 3 times a week subcutaneously for long duration. From 2011 to 2017, the disease process has remained lesion was controlled without any progression.

Discussion

Cutaneous melanoma is an unpredictable tumor. In spite of its outer localization it is often poorly diagnosed at an early stage. Cutaneous melanoma makes up rather a small group of all kinds of skin cancer. Subungual melanoma occurs rarely (8). Clinical symptoms of subungual melanoma are not well-known (9). The average age of patients with cutaneous melanoma is 56 years (10). Such localization correlates with sex and age to a certain extent. Melanoma in the area of fingers in coexistence with the secondary infection can be confused with granuloma (11).

In the presented case it was difficult to differentiate the diagnosis at the stage of giving medical aid in the local polyclinic. Cutaneous melanoma symptoms coexisted with the injury inflammation, rare localization and rare occurrence in the clinical practice of the surgeon in the local polyclinic. After the appearance

of metastases in the left axillary lymph nodes, the patient was given a combined surgical treatment which included amputation of the left thumb and dissection of the left axillary lymph node basin.

In cases when a clinical diagnosis is not possible dermatoscopy can be used simply to confirm the clinical diagnosis (12).

Conclusion

The combination of the inflammatory process, subsequent to nail plate injury made it difficult to make the diagnosis at an early stage although to the current time it has not led to. But it unfavourable consequences.

The case of subungual melanoma combined with the inflammatory process and injury is rather rare. Primary care physicians should follow 'the chaos and clues' in pigmented and 'prediction without pigment' algorithm in non-pigmented skin lesions in assessing patients with suspicious pigmented lesions using a dermatoscope. The 'chaos and clues' and 'prediction without pigment' algorithms lead to a careful examination for clues to subungual melanoma. Skilled use of these algorithms helps to increase diagnostic accuracy for both melanocytic and non-melanocytic skin malignancies (13).

References

1. Apalla Z, Lallas A, Sotiriou E, Lazaridou E, Ioannides D. Epidemiological trends in skin cancer. Dermatol. Pract. Concept. Published online first: April 30, 2017. DOI: 10.5826/dpc.0702a01.
2. Ma J, Guo W, Li C. Ubiquitination in melanoma pathogenesis and treatment. Cancer Med. Published online first: May 23, 2017. DOI: 10.1002/cam4.1069.
3. Satheesha TY, Satyanarayana D, Prasad MNG, Dhruve KD. Melanoma Is Skin Deep: A 3D Reconstruction Technique for Computerized Dermoscopic Skin Lesion Classification. IEEE J Transl Eng Health Med. Published online first: January 16, 2017. DOI: 10.1109/JTEHM.2017.2648797.
4. Brochez L, Verhaeghe E, Bleyen L, Naeyaert JM. Diagnostic ability of general practitioners and dermatologists in discriminating pigmented skin lesions. J Am Acad Dermatol 2001; 4(6): 979-86.
5. Carli P, De Giorgi V, Crocetti E, Caldini L, Ressel C, Giannotti B. Diagnostic and referral accuracy of family doctors in melanoma screening: effect of a short formal training. Eur. J Cancer Prev 2005; 14(l): 51-55.
6. Cassileth BR, Clark WH Jr, Lusk EJ, Frederick BE, Thompson CJ, Walsh WP. How well do physicians recognize melanoma and other problem lesions? J Am Acad Dermatol 1986; 14 (4): 55-60.
7. McGee R, Elwood M, Adam H, et al. The recognition and management of melanoma and other skin lesions by general practitioners in management of melanoma and other skin lesions by general practitioners in New Zealand. N Z Med J 1994; 107(982): 287-90.
8. Haugh AM, Zhang B, Quan VL, et al. Distinct Patterns of Acral Melanoma Based on Site and Relative Sun Exposure. J Invest Dermatol. Published online first: September 1, 2017. DOI: 10.1016/j.jid.2017.08.022.
9. Halteh P, Scher R, Artis A, Lipner S. Assessment of Patient Knowledge of Longitudinal Melanonychia: A Survey Study of Patients in Outpatient Clinics. J. Skin Appendage Disord 2016; 2: 156-161. DOI: 10.1159/000452673.
10. Sinno S., Wilson S., Billig J., Shapiro R., Choi M. Primary melanoma of the hand: An algorithmic approach to surgical management. Published online first: January 7, 2015. DOI: 10.3109/2000656X.2015.1053396.
11. Silva-Feistner M, Ortiz E, Alvarez-Véliz S, Wortsman X. Amelanotic Subungual Melanoma Mimicking Telangiectatic Granuloma: Clinical, Histologic, and Radiologic Correlations. J Actas Dermosifiliogr. Published online first: May 2, 2017. DOI: 10.1016/j.adengl.2017.07.007.
12. Rosendahl C, Cameron A, Tschandl Ph, Bulinska A, Zalaudek I, Kittler H. Prediction without Pigment: a decision algorithm for non-pigmented skin malignancy. Dermatol. Pract. Concept 2014; 4(1): 9: 59-66. Published online first: January 31, 2014. DOI: 10.5826/dpc.0401a09.
13. Rosendahl C, Cameron A, McColl I, Wilkinson D. Dermatoscopy in routine practice – 'chaos and clues'. Australian Family Physician 2012; 41(7): 482-487.

Correspondence:
Evgeny Yurievich Neretin,
Doctoral Student of N.N. Blokhin Russian
Cancer Research Center, Cancer specialist of the Consultation Department, Samara Regional Clinical Oncological Dispensary
E-mail: pretty.step@bk.ru

EUR. J. ONCOL.; Vol. 23, n. 2, pp. 112-116, 2018 © Mattioli 1885

Primary diffuse large B-cell non-Hodgkin lymphoma of the breast in an elderly woman: case report and review of literature

Achille Panetta[1], *Marco Masina*[2], *Silvia Gambini*[1], *Vida Pajetta*[1], *Vincenzo Arigliano*[1], *Roberto Maccaferri*[1], *Massimo Fedele*[1], *Cesare Calandri*[1]

[1] UOSD di Oncologia Territoriale, Azienda USL di Bologna (Italy); [2] UO di Geriatria, Azienda USL di Bologna (Italy)

Summary. Primary non Hodgkin's lymphomas of the breast is a rare disease representing 0.38-0.70% of all non Hodgkin's lymphomas (NHL), 1.7-2.2% of all extranodal NHL and only 0.04-0.5% of all breast tumors. We report a case of an old woman with a primary diffuse large B-cell NHL of the breast who is still alive and disease free after 10 years since the end of treatment with a R-CHOP regimen followed by radiotherapy on residual disease. A review of the literature is also presented.

Key words: breast, primary non Hodgkin lymphoma

Introduction

Primary non-Hodgkin lymphoma of the breast is a rare disease, which represents about 0.38 to 0.70% of all NHL, 1.7 to 2.2% of all cases of extranodal NHL and only 0,04-0.5% of all breast cancers (1-6).

In literature about 700 cases of primary breast NHL (7, 8) have been reported so far. However their incidence is growing. Aviles (9) recently presented a review of 96 patients and the International Extranodal Lymphoma Study Group (IELSG) registered 204 cases (10). Female are the most affected, being primary male breast involvement reported only in rare cases (4, 11,12). The incidence peaks in the fifth and sixth decade of life (5, 8,13).

In 1972, Wiserman and Liao (14) have established the fundamental criteria for the diagnosis of primary lymphoma of the breast: 1) removal of breast tissue enough for a proper pathologic evaluation; 2) close association between breast tissue and lymphomatous infiltrate; 3) exclusion of systemic involvement of lymphoma or previous extra mammalian lymphoma. The ipsilateral axillary lymphadenopathy does not exclude the diagnosis of primary lymphoma of the breast. Although lymphomas represent the largest group of secondary breast cancers, a secondary involvement of the breast in an elsewhere arising lymphoma is a rare occurrence (15).

The most common histological type of primary breast lymphoma is B-cells. T Lymphoma is more rare and anaplastic large T-cell Lymphomas have a higher incidence in women with silicone implants (16-19). The diffuse large B-cell type is the most common B-cell lymphoma, constituting up to 53% of cases (20).

A mucosal-associated lymphoid tissue neoplasm (MALToma) is less frequently observed despite its incidence has increased in recent years since the first description of Lamovec and Jancar in 1987 (21). Burkitt's lymphoma is rare, typically occurs in young women and shows a very aggressive course (16).

Since the first description in 1893 there have been substantial advances in diagnostics and therapeutics of the primary breast NHL. However many controversial issues are still present, mainly concerning the diagnostic and therapeutic conduct. This paper describes the case of a patient with a primitive breast NHL and pre-

sents a review of the most controversial aspects in the diagnostic and therapeutic field.

Case report

In July 2005, a female patient aged 77, came to our attention because of a massive swelling in the upper-outer quadrant of the right breast which had lasted for several weeks (Figure 1).

Family and physiological anamnesis showed no risk factors for breast cancer. Clinical anamnesis recorded no major diseases. On clinical examination, our patient had a massive swelling of an area of about cm 9 per cm 6 which adhered to the underlying plans located in the outer quadrants of the right breast. Overlying skin looked hyperemic with an "orange peel" appearance. The clinical features simulated a carcinomatous mastitis.

Mammography and breast ultrasound were not specific. Serum lactate dehydrogenase (LDH) was within normal range. No systemic symptoms were recorded.

The patient underwent a biopsy of the breast lesion. Histological examination showed a lymphoma consisting of diffuse large peripheral B lymphocytes, immunologically typed as CD20+.

Total body computed tomography and CT-PET showed no further localization of lymphoma. Bone marrow biopsy was negative for systemic involvement.

The patient was administrated with 6 cycles of chemo-immunotherapy, according to the R-CHOP scheme (Rituximab - cyclophosphamide, doxorubicin, vincristine, prednisone).

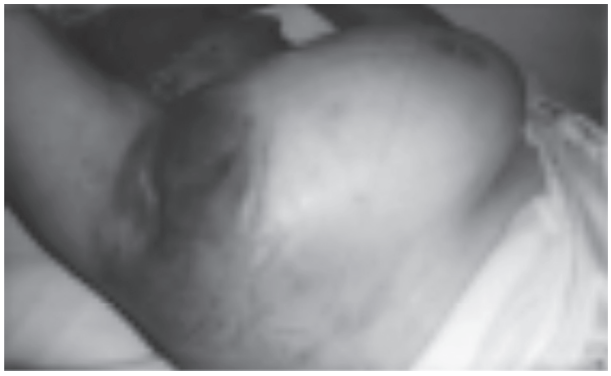

Figure 1. Neoplasms at first clinical examination

A CT/PET at the end of chemo-immunotherapy showed residual disease in the right armpit. Therefore the patient underwent radiation therapy on the right armpit reaching the total dose of 40 Gy in 20 sessions.

A CT/PET revaluation, performed 3 months after completion of radiation therapy, showed a complete remission of malignant disease which it is still maintained about 10 years after the end of treatment.

Discussion and conclusions

Primary NHL of the breast is a rare disease, especially if diagnosis complies with the necessary features as shown above in order to define the primitive mammary origin (14). Because of its features our case can be definitely classified as a primary lymphoma of the breast.

Breast lymphomas are supposed to originate from intramammary lymph nodes and/or periductal and perilobular lymphoid tissue. The low lymphocyte population in this organ justifies the low incidence of cases.

The majority of patients come to clinical observation for the appearance of an asymptomatic unilateral palpable mass. At onset ipsilateral axillary adenopathy is present in 30-40% of cases (14). A higher incidence of lesions in the right breast has been reported but so far no explanation has been proposed for this side preference (22). A bilateral simultaneous presentation at diagnosis is found in approximately 10% of cases (23, 24). Systemic symptoms such as fever, weight loss and night sweats are uncommon findings (2).

The primary lymphoma of the breast is difficult to differentiate from classic carcinoma in preoperative diagnostic phase. Mammography and breast ultrasound are not specific for the diagnosis of lymphoma. Therefore, the diagnosis of breast lymphoma can be obtained performing a cytological examination, for which literature reports a diagnostic accuracy of 86% (25). Greatest diagnostic accuracy is achieved with a needle biopsy (core biopsy) or excisional biopsy. Regardless of histologic type, the lymphomatous cells tend to widely affect the breast parenchyma with infiltration of ductal-lobular structures that are recognizable only in the periphery of the lesion.

After a diagnosis of primary lymphoma of the breast has been histologically confirmed, it is mandatory to perform an accurate staging of the disease for prognostic and therapeutic purposes.

Our patient was subjected to common laboratory tests (including the dosage of lactate dehydrogenase), to total body computed tomography and CT/PET, bone marrow biopsy, all of which were negative for systemic involvement.

The classification of Ann Arbor is the most frequently used in primary NHL of the breast (Table 1). However the International Prognostic Index (Table 2) has proved to be prognostically more accurate than the Ann Arbor staging, allowing the identification of different prognostic sub-groups within the same histological variety (26).

At present a uniform approach to the treatment of primary breast lymphoma is not available. Mastectomy has no indication since lymphomas are in most cases highly chemo and radioresponsive. The treatment is that of systemic lymphomas of the same histological type. Low degree malignant Lymphomas can be treated with a simple excisional biopsy and/or radiotherapy. High-grade malignant lymphoma should be treated with chemotherapy with or without radiation therapy.

Patients with diffuse large B-cell, as in this case, have to start with anthracycline-containing chemotherapy associated to rituximab. The randomized study of the French Groupe d'Etudes des Lymphomes de l'Adulte (GELA) (27) compared 8 cycles of CHOP (cyclophosphamide, adriamycin, vincristine, prednisone) and 8 cycles of CHOP + rituximab (R-CHOP) in 399 patients aged between 60 and 80 years. After a follow-up of 10 years, the addition of rituximab to the CHOP scheme has improved progression-free survival and overall survival of 6%, and disease-free survival for patients with complete remission of 22%. Their conclusions is similar to that of the Mayo

Table 1. Ann Arbor staging for lymphomas[1]

Stage I	Involvement of a single lymph node region (I) or lymphoid structure (eg, spleen, thymus, Waldeyer's ring) or extranodal structure (I_E)
Stage II	Involvement of two or more lymph node regions on the same side of the diaphragm (II) or involvement of limited contiguous extralymphatic organ or tissue (II_E).
Stage III	Involvement of lymph node regions on both sides of the diaphragm (III) which may include the spleen (III_S) and/or limited contiguous extralymphatic organ (III_E) or site (III_{ES})
Stage IV	Multiple or disseminated foci of involvement of extralymphatic organs or tissues and/or lymphatic involement

[1] All stages are further divided on the basis of the absence (A) or presence(B) of the following systemic symptoms: fever >38°C, drenching sweats and/or unexplained weight loss of greater than 10% of body weight over 6 months). Itching alone is not considered a systemic symptom

Table 2. International Prognostic Index (IPI): adverse risk factors of survival at 5 years

Adverse risk factors: (all patients)	Adverse risk factors: patients younger than 60 years of age
Age >60 years	Stage III/ IV (Ann Arbor)
Stage III/IV (Ann Arbor)	Performance status ≥1 (ECOG)
Performance status ≥2 (ECOG)	Serum LDH >normal
Number of Extranodal site >1	

IPI (all patients)	IPI score	Complete Response (%)	Relapse-Free Survival at 5 years (%)	Overall Survival at 5 years (%)
Low	0 o 1	87	70	73
Low intermediate	2	67	50	51
High intermediate	3	55	49	43
High	4 o 5	44	40	26

Clinic group (28), in which the addition of rituximab, administered simultaneously or successively to CHOP, provides a benefit of 30% in terms of progression-free survival at approximately two years. Therefore, in absence of contraindications such as severe heart problems, the use of R-CHOP regimen is strongly recommended in all patients with diffuse large B-cell.

Radiotherapy is indicated when PET scan at the end of R-CHOP chemo-immunotherapy shows a single site residual disease that can be irradiated, as in the reported case. The Canadian group retrospective evaluation (29) of 196 patients treated with 6 cycles of R-CHOP and radiotherapy to PET residues confirmed the advantage of that regimen. Patients who were irradiated showed the same progression free rate at 3 years as patients in complete remission (PET negative) after R-CHOP. The efficacy of R-CHOP regiment plus radiotherapy on residual disease in old patients is also confirmed in our woman who is still alive and free from progression 10 years after completing the treatment.

References

1. Freeman C, Berg JW, Cutler SJ. Occurrence and prognosis of extranodal lymphomas.Cancer 1972; 29: 252-260.
2. Giardini R, Piccolo C, Rilke F. Primary Non-Hodgkins Lymphomas of the female breast. Cancer 1992; 69: 725-35.
3. Bobrow LG, Richards MA, Happerfield LC, Diss TC, Isaacson PG, Lammie GA, Millis RR. Breast lymphomas: a clinic pathologic review.Hum Pathol 1993; 24: 274-278.
4. Arber DA, Simpson JF, Weiss LM, Rappaport H. Non-Hodgkin's lymphoma involving the breast. Am J Surg Pathol 1994; 18: 288-295.
5. Brogi E, Harris NL. Lymphomas of the breast: pathology and clinical behavior.Semin Oncol1999; 26: 357-364.
6. Sokolow T, Shimonov M, Blickstein D, Nobel M, Antebi E. Primary lymphoma of the breast. Unusual presentation of breast cancer. Eur J Surg 2000; 166: 390-393.
7. Avenia N, Sanguinetti A, Cirocchi R, et al. Primary breast lymphomas: a multicentric esperienze. World J Surg Oncol 2010; 8: 53-56.
8. Uesato M, Miyazawa Y, Gunji Y, Ochiai T. Primary Non-Hodgkin's lymphoma of the breast: report of a case with special reference to 380 cases in Japanese literature. Breast Cancer 2005; 12:154-158.
9. Avilés A, Delgado S, Nambo MJ, Neri N, Murillo E, Cleto S. Primary breast lymphoma: results of a controlled clinical trial. Oncology 2005; 69: 256-60.
10. Ryan G, Mantelli G, Kuper-Hommer M, Tsang R, Pruneri G, Yuen K, Roos D, Lennard A, Devizzi L, Crabb S, Hossfeld D, Pratt G, Dell'Olio M, Choo SP, Bociek RG, Radford J, Lade S, Gianni AM, Zucca E, Cavalli F, Seymour JF. Primary diffuse large B-cell lymphoma of the breast. Prognostic factors and outcome of a study by the International Extranodal Lymphoma Study Group.Ann Oncol 2008; 19: 233-241.
11. Tanino M, Tatsuzawa T, Funada T, Nakajima H, Sugiura H, Odashima S. Lymphosarcoma of the male breast. Breast 1984; 10: 13-15.
12. Murata T, Kuroda H, Nakahama T, Goshima H, Shiraishi T, Yatani R. Primary non-Hodgkin malignant lymphoma of the male breast. Jpn J Clin Oncol 1996; 26: 243-247.
13. Domchek SM, Hecht JL, Fleming MD, Pinkus GS, Canellos GP. Lymphomas of the Breast. Primary and Secondary Involvement. Cancer 2002; 94: 6-13
14. Wiseman C, Liao KT. Primary lymphoma of the breast. Cancer 1972; 29: 1705-1712.
15. Hajdu SI, Urban JA. Cancer metastatic to the breast. Cancer 1972; 29: 1691-1696.
16. Jeon HJ, Akagi T, Hoshida Y, Hayashi K, Yoshino T, Tanaka T, Ito J, Kamei T, Kawabata K. Primary non-Hodgkin malignant lymphoma of the breast. An immunohistochemical study of seven patients and literature review of 152 patients with breast lymphoma in Japan. Cancer 1992; 70: 2451-9.
17. Mattia AR, Ferry JA, Harris NL. Breast lymphoma: a B-cell spectrum including low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Am J Surg Pathol 1993; 17: 574-587.
18. Lim HJ, Cho KR, Kim I, et al. Primary peripheral T-cell lymphoma of the breast: radiologic and pathologic findings. J Breast Cancer 2010; 13(3): 318-322.
19. De Jong D, Vasmel WL, Weng A, et al. Anaplastic large-cell lymphoma in women with breast implants. JAMA 2008; 300: 2030.
20. Jennings WC, Baker RS, Murray SS, Howard A, Parker DE, Peabody LF, Vice HM, Sheehan WW, Broughan TA. Primary Breast Lymphoma. The Role of Mastectomy and the Importance of Lymph Node Status. Ann Surg 2007; 245: 784-789.
21. Lamovec J, Jancar J. Primary malignant lymphoma of the breast. Lymphoma of the mucosa-associated lymphoi tissue. Cancer 1987; 60: 3033-3041.
22. Cohen PL, Brooks JJ. Lymphomas of the breast: a clinicopathologic and immunohistochemical study of primary and secondary cases. Cancer 1991; 67: 1359-1369.
23. Topalovski M, Cristan D, Mattson JC. Lymphoma of the breast: a clinicopathologic study of primary and secondary cases. Arch Pathol Lab Med 1999; 123: 1208-1218.
24. Pruthi S, Stafyla VK, Phillips SW, et al. Primary mammary (non Hodgkin) lymphoma presenting as locally advanced breast cancer. Mayo Clin Proc 2004; 79(10): 1310-1314.
25. Duncan VE, Reddy VV, Jhala ND, et al. Non Hodgkin's lymphoma of the breast: a review of 18 primary and secondary cases. Ann Diagn Pathol 2006; 10: 144-148.

26. Shipp e al. A predictive model for aggressive non-Hodgkin's lymphoma – The International Non-Hodgkin's Lymphoma Prognostic Factors Project. N Engl J Med 1993; 329: 987-994.
27. Coiffier B, Thieblemont C, Van Den Neste E, et al. Long-term outcome of patients in the LNH-98.5 trial, the first randomized study comparing rituximab-CHOP to standard CHOP chemotherapy in DLBCL patients: a study by the Groupe d'Etudes des Lymphomes de l'Adulte. Blood 2010; 116: 2040-2045.
28. Habermann TM, Weller EA, Morrison VA, et al. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. J Clin Oncol 2006; 24: 3121-3127.
29. Sehn L, Klasa R, Shenkier T. Long-term experience with PET-guided consolidative radiation therapy in patients with advanced stage diffuse large B-cell lymphoma treated with R-CHOP. Hematological Oncology 2013; 31(S1): 137.

Correspondence:
Achille Panetta, MD
UOSD di Oncologia Territoriale, Azienda USL di Bologna
Via Marconi 35 - 40010 Bentivoglio, Bologna (Italy)
Tel. +39-051-6644221
Fax +39-051-6644030.
E-mail: a.panetta@ausl.bologna.it