FIZIOLOGIA
physiology

ACCREDITED BY CNCSIS - B+CATEGORY - CODE 240

http://journals.indexcopernicus.com/karta.php?action=masterlist&id=4929
http://www.ebscohost.com/titleLists/a9h-journals.pdf

Publication data: Fiziologia (Physiology) is issued quarterly
Subscription rates: Subscriptions run a full calendar year. Prices are give per volume, surface postage included.
Personal subscription: Romania - 100 RON, Outside Romania - 35$ (must be in the name of, billed to, and paid by an individual. Order must be marked "personal subscription")
Institutional subscription: 50$ (regular rate)
Single issues and back volumes: Information on availability and prices can be obtained through the Publisher.
Change of address: Both old and new address should be stated and send to the subscription source.
Bibliographic indices: We hope this journal will be regularly listed in bibliographic services, including "Current Contents".
Book Reviews: Books are accepted for review by special agreement.
Advertising: Correspondence and rate requests should be addressed to the Publisher.

1. FOR SUBSCRIPTION ADDRESS

HVB Bank TIMISOARA
RO 21 BACX 0000000218508250

TIMISOARA – ROMANIA
PENTRU REVISTA
„FIZIOLOGIA – PHYSIOLOGY"

2. CORRESPONDENCE SHOULD BE ADDRESSED TO THE CHIEF EDITOR

PROF. DR. FRANCISC SCHNEIDER
PO BOX 135
300024 – TIMISOARA – ROMANIA
e-mail: carmen.tatu@umft.ro

Editura **EUROSTAMPA**
Tel./fax: 0256-204816
ISSN 1223 – 2076

Instructions to Authors

Submission: Only original papers in English are considered and should be sent to:

Prof. dr. Francisc Schneider
Chief Editor of "Fiziologia"
PO Box 135
300024, TIMISOARA, ROMANIA
Tel./Fax: 40-256/490507

Manuscripts should be submitted in triplicate sets of illustrations (of which one is an original), typewritten doublespaced on one side of the paper, with a wide margin.

Conditions: All manuscripts are subject to editorial review. Manuscripts are received with the explicit understanding that they are not under simultaneous consideration by any other publication. Submission of an article for publication implies the transfer of the copyright from the author to the publisher upon acceptance. Accepted papers become the permanent property of "Fiziologia" (Physiology) and may not be reproduced by any means, in whole or in part, without the written consent of the publisher. It is the author's responsibility to obtain permission to reproduce illustrations, tables, etc. from other publications.

Arrangement:

Title page: The first of each paper should indicate the title (main title underlined), the authors' names, and the institute where the work was conducted. A short title for use as running head is also required.

Keywords: for indexing purposes, a list of 3-10 keywords in English and Romanian is essential.

Abstract: Each paper needs abstract and title in Romanian and English language, fonts size 9, Arial Narrow.

Bady text: fonts size 10, Arial Narrow.

Small type: Paragraphs which can or must be set in smaller type (case histories, test methods, etc.) should be indicated with a „p" (petit) in the margin on the left-hand side.

Footnotes: Avoid footnotes. When essential, they are numbered consecutively and typed at the foot of the appropriate page, fonts size 8, Arial Narrow.

Tables and illustrations: Tables (numbered in Roman numerals) and illustrations (numbered in Arabic numerals) should be prepared on separate sheets, fonts size 9, Arial Narrow. Tables require a heading, and figures a legend, also prepared on a separate sheet. For the reproduction of illustrations, only good drawings and original photographs can be accepted; negatives or photocopies cannot be used. When possible, group several illustrations on one block for reproduction (max. size 140x188 mm) or provide crop marks. On the back of each illustration indicate its number, the author's name, and article title. Colour illustration are reproduced at the author's expense.

References: In the text identify references by Arabic figures, (in brackets), fonts size 9, Arial Narrow. Material submitted for publication but not yet accepted should be noted as "unpublished data" and not be included in the reference list. The list of references should include only those publications which are cited in the text. The references should be numbered and arranged alphabetically by the authors' names. The surnames of the authors followed by initials should be given. There should be no punctuation signs other than a comma to separate the authors. When there are more than 3 authors, the names of the 3 only are used, followed by "et al". abbreviate journal names according to the Index Medicus system. (also see International Committee of Medical Journal Editors: Uniform Requirements for manuscripts submitted to biomedical journals. Ann Intern Med 1982; 96: 766 – 771).

Examples:

(a) Papers published in periodicals: Kauffman HF, van der Heide S, Beaumont F, et al: Class-apecific antibody determination against Aspergillus fumigatus by mean of the enzyme-linked immunosorbent assay. III. Comparative study: IgG, IgA, IgM, ELISA titers, precipitating antibodies and IGE biding after fractionation of the antigen. Int Arch Allergy Appl Immunol 1986; 80: 300 – 306.

(b) Monographs; Matthews DE, Farewell VT: *Using and Understanding Medical Statistics*. Basel, Karger, 1985.

(c) Edited books: Hardy WD Jr, Essex M: *FeLV-inducted feline acquired immune deficiency syndrome: A model for human AIDS;* in Klein E(ed): Acquired Immunodeficiency Syndrome. Prog Allergy, Busel, Karger, 1986, vol 37, 353 – 376.

Full address: The exact postal address complete with postal code of the senior author must be given; if correspondence is handled by someone else, indicate this accordingly. Add the E-mail address if possible.

Page charges: There is no page charge for papers of 4 or fewer printed pages (including tables, illustrations and references).

Galley proofs: unless indicated otherwise, galley proofs are sent to the first-named author and should be returned with the least possible delay. Alternations made in galley proofs, other than the corrections of printer's errors, are charged to the author. No page proofs are supplied.

Reprints: Order forms and a price list are sent with the galley proofs. Orders submitted after the issue is printed are subject to considerably higher prices. Allow five weeks from date of publication for delivery of reprints.

FIZIOLOGIA
physiology

CONTENTS

CUPRINS

SILENCING OF THE HER2 GENE BY RNA INTERFERENCE INHIBITS PROLIFERATION OF BREAST CANCER CELL LINE SK-BR-3

OANA I. GAVRILIUC[1], FLORINA M. BOJIN[1], ADRIANA ROSCA[2], CALIN A. TATU[2], VIRGIL PAUNESCU[1]

[1]Physiology-Immunology Department, "Victor Babes"University of Medicine and PharmacyTimisoara

[2]Regional Center for Transplant Immunology, Emergency Clinical County Hospital Timisoara

ABSTRACT

Introduction: HER2 overexpression has been reported in approximately 25% of invasive breast cancers, with or without genomic amplification of the gene and is associated with poor disease survival and resistance to chemotherapeutic agents. In this study, we investigated the effect the silencing of c-*erbB2* gene expression by RNA interference would have on the proliferation and viability of breast cancer cell line SK-BR-3, known to express the HER2*neu* protein at very high levels.

Materials and methods: siRNA were delivered into the target cells using the Lipofectamine 2000 reagent. Total RNA was extracted with TRIzol reagent, reverse transcribed to cDNA and cDNA samples were analyzed by qPCR for HER2 and VEGF expression following lipofection. HER2 protein expression was assessed by immunohistochemistry, while VEGF presence in the cell culture supernatants was documented by ELISA. siRNA treated and untreated SK-BR-3 cells were also assayed for cell cycle distribution and induction of apoptosis.

Results: An inhibition of ~ 80% in the expression of the oncogene inhibited the proliferation of breast cancer cells, by blocking their cell cycle in the G0/G1 phase and also resulted in a reduction of VEGF secretion by half. However, we did not observe an increased apoptosis in the siRNA treated breast cancer cells when compared to control untreated cells.

Conclusion: The complex mechanism by which c-*erb*B2 stimulates cancer cell growth and resistance to chemotherapy has not been completely defined and there certainly exist redundant pathways sustaining the proliferation of neoplastic cells.

Keywords: HER2/neu, RNA interference, breast cancer, SK-BR-3

INTRODUCTION

Human tumors demonstrate unique profiles of gene expression, different from normal tissue and from other tumors, resulting in a distinct rewiring of protein pathways which drive the abnormal proliferation of cancer cells. In turn, the malignant cells will become highly dependent on the rewired pathway that ensures their survival and robustness to certain types of therapeutic attacks. Therefore, a targeted knockdown of an oncogenic pathway should theoretically lead to cell death in malignant cells without affecting the normal tissues (1).

A particularly efficient posttranscriptional silencing of a gene of interest can be achieved by RNA interference (RNAi), which represents a sequence-specific, naturally occurring mechanism of cellular defense and gene regulation that has been converted to a key laboratory technique over the last decade. RNAi relies on the cleavage of a long double-stranded RNA (dsRNA) into 21-23 base pair molecules termed small interfering RNAs (siRNA) by an intracellular endonuclease called Dicer before being loaded into RISC and targeting the specific degradation of an homologous mRNA (2). *In vitro*, RNAi can be induced either by direct transfection of small interfering RNA duplexes (3), which bypass the defense mechanisms against dsRNAs, by endogenously produced noncoding microRNAs, or by introduction of a vector driving the expression of short hairpin RNA (shRNA) that are further processed into siRNA by Dicer. Although the actual use of siRNA for *in vivo* human therapy is still plagued by the lack of a safe and efficient method of delivery to the target tissues and cells, a number of preclinical studies and early clinical studies have established the proof of concept that RNAi could be potentially used in the treatment of a variety of disease, among which cancer is of leading importance (4).

In this report, we addressed a gene that has been shown previously to be relevant or rate-limiting for tumor growth: c-*erbB2*. The *c-erbB2* proto-oncogene encodes the receptor tyrosine kinase HER2*neu* which is responsible for increased proliferation and survival of the primary tumor, but also plays a role in the motility of intravasating and extravasating tumor cells (5). HER2 overexpression has been reported in approximately 25% of invasive breast cancers, with or without genomic amplification of the gene (6) and is associated with poor disease survival and resistance to chemotherapeutic agents (7,8). Monoclonal antibodies designed to dowregulate HER2 expression in cancer cells, currently used in clinical therapy, have shown a number of limitations in their ability to modulate HER2 function (9), while employing antisense oligonucleotides for RNAi-mediated inhibition of HER2*neu* demonstrated better regulation of HER2 expression and induction of apoptosis in cancer cells (10,11). We investigated the effect a silencing of c-*erbB2* gene expression would have on the proliferation and viability of breast cancer cell line SK-BR-3, known to express the

Received August 12th 2011. Accepted September 15th 2011. Address for correspondence: Oana Gavriliuc, PhD, Immunology Department, "Victor Babes" University of Medicine and Pharmacy Timisoara, Spl. Tudor Vladimirescu no. 14A, phone/fax: +40256490507, e-mail: oana_gav@yahoo.com

HER2*neu* protein at very high levels.

MATERIALS AND METHODS

Cell culture

Breast cancer cell line SK-BR-3 was obtained from American Type Culture Collection (Manassas, VA). Media and FCS were purchased from Sigma (St. Louis, MO, USA) and Gibco-BRL (Invitrogen, Carlsbad, CA, USA), respectively. The cell line was maintained in culture according to supplier's instructions, in McCoy's 5a medium with 1.5 mM L-glutamine, 3.0 g/L glucose, 2.2 g/L sodium bicarbonate and 10% FCS.

siRNA

siRNA were designed according to Elbashir et al. [12] and purchased from Eurogentec (Seraing, Belgium). Three different regions within the c-*erb*B2 (NCBI accession no. NM_001005862) gene were used: siR_1H (positions 861-879), siR_2H (positions 2678-2696), siR_3H (positions 3136-3154). A proprietary Her2-targeted siRNA and fluorescent control siRNA were obtained from SCBT (Santa Cruz, CA). All siRNA contain a 19-bp double-stranded (ds) sequence and symmetric 3' overhangs of two deoxythymidines.

In vitro transfection of siRNA

siRNA were delivered into the target cells using the Lipofectamine 2000 reagent (Invitrogen). Prior to the transfection, the cells were permitted to reach 60-70% confluence, and the complete culture medium was changed to medium without antibiotics 30 minutes before lipofection. Alternatively, cells were transfected in suspension, according to the manufacturer's protocol. siRNA were diluted in serum-free OptiMEM so that the final concentration when added to the cells would be 100 nM. Lipofectamine 2000 was mixed and diluted at the appropriate amount in serum-free OptiMEM. The diluted DNA was mixed with Lipofectamine 2000 in a ratio of 1:1, and the liposomal-DNA complexes formed after a 20- minute incubation at room temperature were added to the cells. We used as a positive control for assessing transfection rate the control siRNA (Fluorescein Conjugate)-A (SCBT). The transfection medium was changed to complete growth medium after 16-20 hours and gene expression was evaluated at different time points. All transfection reagents were purchased from Invitrogen.

qRT-PCR assay of gene expression levels

All RNA samples used in this study were isolated in our laboratory from breast cancer cell lines MDA-MB-231 and MCF-7, mammary tumor and normal tissue, tumor-associated fibroblasts (TAF) and mesenchymal stem cells (MSC). Total RNA was extracted with TRIzol reagent (Invitrogen) following the supplier's instructions. RNA concentration was determined with the ND-1000 spectrophotometer. Sample purity, as indicated by the 260/280 absorbance ratio, was 1.9-2.02. We used 1 ug total RNA for every 20 ul reverse transcription reaction performed with the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Palo Alto, CA). cDNA samples were analyzed by quantitative real-time PCR, using the LightCycler 480 SYBR Green I Master (Roche, Florence, SC, USA) and the following primers: human erbB2 forward 5'-CTGGTGACACAGCTTATGCCCT-3'; reverse 5'- ATCCCCTTGGCAATCTGCA-3'; human VEGF forward 5'-CTACCTCCACCATGCC-AAGT-3' and reverse 5'-TGGTGAGAGATCTGGTTCCC. HPRT1 (hypoxanthine phosphoribosyltransferase 1) was chosen as a suitable reference gene; the primers were HPRT1 forward 5'-CCTGGCGTCGTGATTAGTGAT-3' and reverse 5'-AGACGTTCA-GTCCTGTCCATAA-3' We performed a relative basic quantitation based on the ΔΔCt method with the LightCycler480 Software.

Cell cycle analysis and apoptosis assay

The cell cycle distribution of erbB2 siRNA treated and untreated SK-BR-3 cells was determined by flow cytometry and measured as propidium iodide (PI) fluorescence intensity on a FACSCalibur (BD Bioscience, Heidelberg, Germany). At the specified time following siRNA transfection, cells were harvested and stained using the CycleTEST PLUS DNA reagent kit (BD Bioscience). Analysis was performed on 10 000 events for each sample. The percentages of cells within the G0/G1, S and G2/M phases of the cell cycle were determined using the CellQuest Pro software.

To assay for apoptosis induction following the inhibition of erbB2 in the breast cancer cell line SK-BR-3, cells were stained with 10 uL FITC-conjugated Annexin V (Miltenyi Biotec, Bergisch Gladbach, Germany) and 10 uL propidium iodide (PI, 1 ug/mL; BD Bioscience). The cells were subsequently analyzed by flow cytometry.

Her2/neu protein expression

To determine inhibition of the protein product of the erbB2 gene following RNAi, Her2/neu expression on cell membrane was quantified by flow cytometry. Cells were harvested by trypsinization at the specified time points, washed twice with PBS and stained for Her2/neu with a fluorescein-conjugated anti-erbB2 Affibody Molecule (Abcam, Cambridge, MA) directed against the extracellular domain of human erbB2. At least 20,000 cells from each staining were analyzed on a FACSCalibur flowcytometer (BD Bioscience) equipped with the CellQuest Pro software.

We also assessed the Her2/***neu*** protein level in SK-BR-3 cells by immunohistochemical staining. Transfected cells were spotted on CytoSpin coated slides (Thermo Scientific, Bremen, Germany) by cytocentrifugation, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. The primary polyclonal rabbit anti-human c-***erb***B2 antibody was purchased from DakoCytomation (Glostrup, Denmark) and was tested for human specificity and cross-reactivity. Staining protocol continued with secondary biotinylated antibody binding, fuchsin substrate addition, and hematoxylin counterstaining of the nuclei (LSAB+ System-AP, Dako) following the manufacturer's procedures. Microscopy analysis was performed on a Nikon Eclipse E800 microscope.

VEGF ELISA

To determine VEGF secretion in the cell culture medium,

we collected culture supernatants at 48 hours following transfection, filtered through 0.22 μm strainers and stored them at -80°C. The undiluted samples were analyzed with the Quantikine HUman VEGF Immunoassay Kit (R&D Systems, Minneapolis, MN, USA), as per manufacturer's instructions. We compared supernatants collected from equal numbers of cells which were lipofected and VEGF concentration in the supernatant was expressed as pg/mL.

Statistical analysis

All statistical tests and values were calculated with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± s.e.m. Groups were compared using one-way ANOVA for normally-distributed data or the Mann-Whitney *U* test for nonparametric values. P<0.05 was considered to be significant.

RESULTS

Expression of c-erbB2 mRNA and protein in breast cancer cell line SK-BR-3

We documented c-*erb*B2 mRNA expression by quantitative RT-PCR in three breast cancer cell lines, mammary tumor tissue and tumor-associated fibroblasts (TAFs), as well as mesenchymal stem cells (MSCs) as normal cell culture counterparts, and normalized the results to c-*erb*B2 expression in normal tissue samples (Figure 1 **A**). As previously reported in the literature (13,14), HER2/*neu* expression is highest for SK-BR-3 cells, while MCF-7 and MDA-MB-231 expressed less to no HER2/*neu* protein. Mesenchymal stem cells expressed almost undetectable levels of c-*erb*B2 mRNA, when mammary tumor tissue and TAFs showed somewhat increased expression of c-*erb*B2**.** High levels of HER2/*neu* transcripts in the breast cancer line SK-BR-3 were correlated with an abundant expression of the protein on cell membrane, as revealed by immunocytochemistry (Figure 1 **B**).

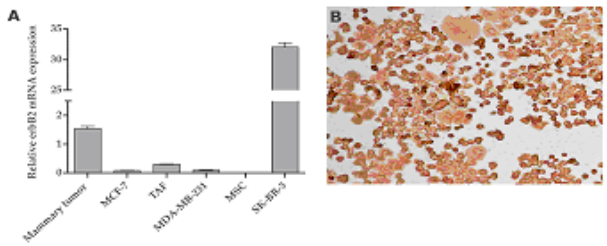

Fig.1. Expression of c-erbB2 mRNA in breast cancer cell lines MCF-7, MDA-MB-231 and SK-BR-3, mammary tumor tissue, TAFs and MSCs. Normalized to normal tissue RNA. Breast cancer cells SK-BR-3 overexpress c-erbB2 by more than 30-fold (A, quantitative PCR analysis). B Positive immunocytochemical staining for c-erbB2 in SK-BR-3 cell line. Optical microscopy, 100x magnification.

Inhibition of c-erbB2 mRNA and protein expression by specific siRNA

Three siRNA duplexes were designed against the c-*erb*B2 oncogene and we examined the ability of the constructs, individually or combined in equimolar amounts, to downregulate HER2/*neu* transcription in the HER2-overexpressing breast carcinoma SK-BR-3. Alternatively, we purchased an siRNA duplex targeting HER2/*neu* from SCBT. We transfected the cells with the Lipofectamine 2000 reagent and 100 nM siRNA duplexes and determined oncogene expression by quantitative real-time PCR at 24 hours following lipofection. Cells transfected with fluorescein-conjugated control siRNA served as both control for lipofection and for inhibition.

Although all siRNA duplexes resulted in some level of inhibition ranging from 21% to 82% (Figure 2 **A**) compared to oncogene expression in the mock transfected cells, the most pronounced reduction of mRNA levels were observed for siR_1H against Her2/*neu*. Simultaneous delivery of a pool of all three siRNA did not improve efficiency of inhibition significantly. Consequently, for all further inhibition experiments, we employed siR_1H siRNA.

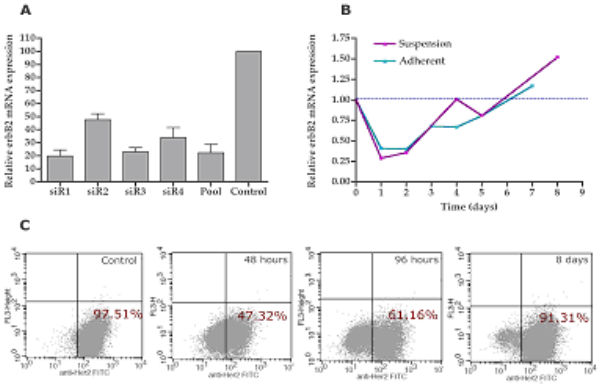

Fig. 2. A Expression of c-erbB2 mRNA at 24 hours from siRNA lipofection of SK-BR-3 c-erbB2 overexpressing breast cancer cells. The highest efficiency of mRNA inhibition was observed for siR_1H , when c-erbB2 expression was reduced by 77% . B mRNA c-erbB2 expression was determined and plotted every 24 hours post-silencing, showing the step-wise return to previously detected levels. siRNA lipofection of either adherent or trypsinized SK-BR-3 cells did not influence treatment outcome. C Flow cytometry analysis of HER2neu expression at 48, 96 hours and 8 days in siRNA treated and control SK-BR-3 cells.

Next, we assessed the time frame for which the siRNA siR_1H is capable of downregulating c-*erb*B2 mRNA and protein expression to therapeutically useful levels and documented the return of c-*erb*B2 expression to the levels detected before RNAi. Transcription of c-*erb*B2 was maintained at below 50% of that in untreated cells for the first 48 hours, quickly increasing afterwards to previous expression levels at 6 days and even overcompensating with higher levels than before transfection when measured at 8 days post-silencing (Figure 2 **B**). Flow cytometry analysis of HER2 levels in transfected SK-BR-3 showed decreased protein levels to less than 50% at 48 hours following inhibition. The level of HER2 protein inhibition regressed slower than mRNA levels, with 61.6% of the transfected cells still expressing significantly low HER2 96 hours post-treatment (Figure 2 **C**). At the last time point considered, 8 days, there was still a small population of cells that did not regain previous HER2 expression.

Furthermore, we examined by immunocytochemistry and confirmed c-*erb*B2 protein knockdown at 48 hours after siR_1H delivery in the SK-BR-3 line (Figure 3).

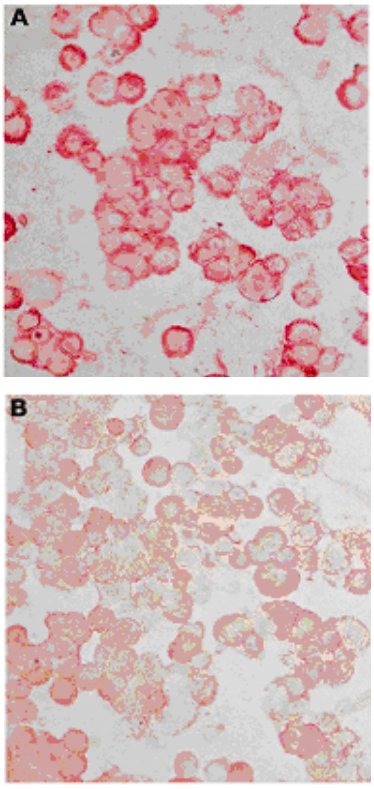

Fig. 3. Immunocytochemical analysis of SK-BR-3 cells of Her2/neu protein. Untreated control cells (A) express the protein at very high levels (3+), while cells treated with 100 nM siR_1H (B), still express HER2/neu ubiquitously, but at significantly lower levels. Optical microscopy, magnification 400x.

Her2/neu inhibition reduces VEGF secretion in breast cancer cell line SK-BR-3

We determined the presence of pro-angiogenic vascular endothelial growth factor (VEGF) in the cell culture medium of SK-BR-3 cells after siRNA inhibition of the oncogene of interest. A significantly lower concentration of VEGF was correlated with c-*erb*B2 inhibition and downregulation of VEGF mRNA expression, as detected by quantitative RT-PCR (Figure 4 **A**).

Cell cycle and viability of HER2neu-silenced SK-BR-3 cells

SK-BR-3 cells treated with c-*erb*B2 siRNA showed growth inhibition, morphological changes and an inability to form a confluent monolayer when examined under an inverted microscope (data not shown). When stained with PI and analyzed for cell cycle distribution, HER2-silenced cells displayed gowth arrest and an accumulation of cells in the G0/G1 phase of the cell cycle (Figure 4 **B**) at 48 and 96 hours post-transfection when compared with the untreated control cells. However, at 8 days following inhibition, the cell cycle distribution was similar to control cells.

We tried to determine whether there was also an increase in the percentage of apoptotic cell in the SK-BR-3 cells transfected with HER2*neu* siRNA, but that was not case as the apoptotic and dead cell populations recognized by characteristic Annexin V- positive, PI negative and Annexin V - positive, PI positive staining, respectively, did not differ significantly from controls.

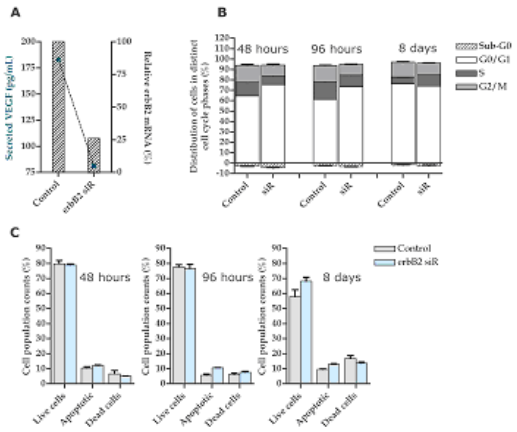

Fig. 4. A VEGF presence in SK-BR-3 cell culture supernatant (pg/mL; left y-axis, connecting dots plot), determined by ELISA immunoassay, following siRNA inhibition of c-erbB2. Supernatants were collected at 48 hours from transfection, from identical numbers of cells, incubated for 24 hours in normal cell growth medium. VEGF transcription, detected by qRT-PCR (right y-axis, bar plot). B Cell cycle distribution determined by flow cytometry analysis of cells treated with propidium iodide (PI). Sub-G0 phase indicates apoptotic cells. C Quantitative analysis of apoptosis by Annexin V and propidium iodide (PI) staining.

DISCUSSION

We studied the effects of HER2 siRNA transfection on SK-BR-3 cells, a HER2/*neu* overexpressing breast adenocarcinoma cell line. Previously published experiments using RNA interference for silencing c-*erb*B2 expression in human breast cancer cells reported a dose-dependent suppression of HER2 levels and inhibition of growth or induction of apoptosis in the cancer cell lines employed in the study (9-11,15,16). Testing 4 different siRNAs directed against different parts of the -*erb*B2 mRNA sequence, as well as an equimolar mix of these siRNAs, we were able to obtain a significant knockdown of HER2 transcription ranging from 15 to 85%, correlated to a more than 50% decrease of surface protein expression. Three of the siRNA sequences were not published before, while the fourth was purchased from SCBT. We obtained the highest inhibition efficiency in the case of siR_H1, one of our custom designed sequences, which we proceeded to use for all subsequent experiments. As expected in the case of lipofection, a transient transfection method, HER2 levels of gene expression returned to normal in approximately 6 days, with a slight increase above the starting level of transcription at 8 days following the silencing procedure, but we could not find any data in the literature to compare the kinetics of inhibition specifically for the SK-BR-3 cell line for a period longer than 96 hours. The effect of siRNA transfection on HER2 protein presence on the cell surface was more long-lasting, the cells recovering to 90% of the initial protein level at 8 days after inhibition. Although the silencing of the target gene by RNAi was not permanent, one single transfection resulted in levels of inhibition of the oncogene much higher than those reported in the case of monoclonal antibodies (17,18) or antisense oligonucleotides (19).

Breast cancer cells that overexpress HER2 have been shown to produce high levels of the potent pro-angiogenic vascular endothelial growth factor VEGF (20,21). In our present study, we also observed a marked decrease of VEGF level after silencing c-*erb*B2 expression by more than half, compared to untreated SK-BR-3 cells, a very important effect of HER2 RNAi resulting in a multi-targeted tumor control.

One of the most significant physiological impacts of HER2/*neu* inhibition on SK-BR-3 cells was an increased arrest of the siRNA treated cells in the G0/G1 phase of the cell cycle. This finding is consistent with other reports in the literature (10,11), while there is also one study that presented growth arrest at G2/M (9). Down-regulation of c-*erb*B2 would result to decreased phosphorylated Akt, which leads to decreased expression of the cyclin D1 involved in the regulation of G0/G1 cell cycle arrest. Another possible way of explaining G1 arrest would be a decrease of CDK inhibitor p27Kip1 phosphorylation, another downstream target of Akt (22,23). We did not however observe an increased apoptosis in the siRNA treated breast cancer cells when compared to control untreated cells. This might be explained by the very high initial level of HER2 expression in SK-BR-3 cells with even 20 percent of remaining oncogene expression accounting for continued survival of tumor cells. The complex mechanism by which c-*erb*B2 stimulates cancer cell growth and resistance to chemotherapy has not been completely defined and there certainly exist redundant pathways conveying a proliferation stimulus in the neoplastic cells which converge. We intend to investigate next the effects of a longer inhibition of HER2 in the breast cancer cell line SK-BR-3 either by repeated transfection or by using a viral construct capable of long-term silencing of the target gene, in which experimental setting the network of HER2 targets might be more apparent.

Acknowledgements

This work was supported by CNCSIS – UEFISCSU, project number PNII – IDEI 1748/2008 awarded to VP and POSDRU/89/1.5/S/62371 awarded to OIG.

REFERENCES

1. Phalon C, Rao DD, Nemunaitis J. Potential use of RNA interference in cancer therapy. *Expert Rev Mol Med* 2010; 12e26.
2. Latterich M. Rnai advanced methods. illustrated ed. Taylor & Francis; 2008.
3. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide rnas. *Genes Dev* 2001; 15(2): 188-200.
4. Manjunath N, Dykxhoorn DM. Advances in synthetic sirna delivery. *Discov Med* 2010; 9(48): 418-30.
5. Freudenberg JA, Wang Q, Katsumata M, et al. The role of HER2 in early breast cancer metastasis and the origins of resistance to her2-targeted therapies. *Exp Mol Pathol* 2009; 87(1): 1-11.
6. Press MF, Slamon DJ, Flom KJ, et al. Evaluation of HER-2/neu gene amplification and overexpression: Comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. *J Clin Oncol* 2002; 20(14): 3095-105.
7. Esteva FJ, Cheli CD, Fritsche H, et al. Clinical utility of serum HER2/neu in monitoring and prediction of progression-free survival in metastatic breast cancer patients treated with trastuzumab-based therapies. *Breast Cancer Res* 2005; 7(4): R436-43.
8. Morrow PK, Zambrana F, Esteva FJ. Recent advances in systemic therapy: Advances in systemic therapy for her2-positive metastatic breast cancer. *Breast Cancer Res* 2009; 11(4): 207.
9. Choudhury A, Charo J, Parapuram SK, et al. Small interfering RNA (sirna) inhibits the expression of the her2/neu gene, upregulates HLA class I and induces apoptosis of her2/neu positive tumor cell lines. *Int J Cancer* 2004; 108(1): 71-7.
10. Faltus T, Yuan J, Zimmer B, et al. Silencing of the HER2/neu gene by sirna inhibits proliferation and induces apoptosis in HER2/neu-overexpressing breast cancer cells. *Neoplasia* 2004; 6(6): 786-95.
11. Yang G, Cai KQ, Thompson-Lanza JA, et al. Inhibition of breast and ovarian tumor growth through multiple signaling pathways by using retrovirus-mediated small interfering RNA against her-2/neu gene expression. *J Biol Chem* 2004; 279(6): 4339-45.
12. Elbashir SM, Harborth J, Lendeckel W, et al. Duplexes of 21-nucleotide rnas mediate RNA interference in cultured mammalian cells. *Nature* 2001; 411(6836): 494-8.
13. Hartsuiker L, Zeijen NJ, Terstappen LW, et al. A comparison of breast cancer tumor cells with varying expression of the her2/neu receptor by raman microspectroscopic imaging. *Analyst* 2010; 135(12): 3220-6.
14. Xiao Y, Gao X, Maragh S, et al. Cell lines as candidate reference materials for quality control of ERBB2 amplification and expression assays in breast cancer. *Clin Chem* 2009; 55(7): 1307-15.
15. Colomer R, Lupu R, Bacus SS, et al. Erbb-2 antisense oligonucleotides inhibit the proliferation of breast carcinoma cells with erbb-2 oncogene amplification. *Br J Cancer* 1994; 70(5): 819-25.
16. Roh H, Pippin JA, Green DW, et al. HER2/neu antisense targeting of human breast carcinoma. *Oncogene* 2000; 19(53): 6138-43.
17. Brodowicz T, Wiltschke C, Budinsky AC, et al. Soluble HER-2/neu neutralizes biologic effects of anti-her-2/neu antibody on breast cancer cells in vitro. *Int J Cancer* 1997; 73(6): 875-9.
18. Drebin JA, Link VC, Weinberg RA, et al. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. *Proc Natl Acad Sci U S A* 1986; 83(23): 9129-33.
19. Roh H, Pippin J, Drebin JA. Down-Regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. *Cancer Res* 2000; 60(3): 560-5.
20. Le XF, Mao W, Lu C, et al. Specific blockade of VEGF and HER2 pathways results in greater growth inhibition of breast cancer xenografts that overexpress HER2. *Cell Cycle* 2008; 7(23): 3747-58.
21. Loureiro RM, Maharaj AS, Dankort D, et al. Erbb2 overexpression in mammary cells upregulates VEGF through the core promoter. *Biochem Biophys Res Commun* 2005; 326(2): 455-65.
22. Liang J, Zubovitz J, Petrocelli T, et al. PKB/akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med* 2002; 8(10): 1153-60.
23. Zhuang Y, Miskimins WK. Cell cycle arrest in metformin treated breast cancer cells involves activation of AMPK, downregulation of cyclin D1, and requires p27kip1 or p21cip1. *J Mol Signal* 2008; 318.

INHIBITIA EXPRESIEI GENEI HER2 PRIN INTERFERENTA ARN BLOCHEAZA PROLIFERAREA LINIEI TUMORALE MAMARE SK-BR-3

REZUMAT

Introducere: Supraexpresia genei HER2, cu sau fara amplificare genomica, a fost documentata in aproximativ 25% din cazurile de cancer mamar si a fost asociata cu un prognostic nefavorabil si rezistenta la chemoterapie. In acest studiu, am investigat efectul inhibitiei expresiei protooncogenei c-*erbB2* prin interferenta ARN aupra proliferarii si viabilitatii liniei tumorale mamare SK-BR-3, care exprima HER2*neu* la un nivel ridicat.

Materiale si metode: Moleculele de siRNA au transfectate cu ajutorul reactivului Lipofectamine 2000. ARN-ul total a fost extras cu TRIzol, revers-transcris sub forma de ADNc, iar probele de ADNc au fost analizate prin metoda qPCR pentru a determina nivelul de transcriptie a genelor HER2 si VEGF in urma inhibitiei. Expresia proteica de HER2 a fost pusa in evidenta prin colorare imunohistochimica, in timp ce prezenta VEGF in supernatant a fost documentata prin ELISA. Am determinat, de asemenea, distributia ciclului celular si gradul de apoptoza in celulele SK-BR-3 tratate cu siRNA, comparativ cu un control.

Rezultate: O inhibitie de ~80% a expresiei oncogenei a oprit proliferarea celulelor tumorale prin blocarea ciclului celular in faza G0/G1 si a rezultat intr-o reducere accentuata a secretiei de VEGF. Nu am observat, insa, un nivel crescut de apotoza in celule tumorale tratate cu siRNA.

Concluzii: Mecanismul complex prin care gena c-*erb*B2 stimuleaza proliferarea tumorala si rezistenta la chemoterapie nu a fost complet elucidat si, in mod cert, exista cai de semnalizare redundante care sustin proliferarea neoplastica in absenta HER2.

Cuvinte-cheie: HER2/neu, interferenta ARN, cancer mamar, SK-BR-3

BIOGERONTOLOGY TODAY. PART I. 61ST ANNUAL SCIENTIFIC MEETING OF THE BRITISH SOCIETY FOR RESEARCH ON AGEING - BSRA & 14TH CONGRESS OF THE INTERNATIONAL ASSOCIATION OF BIOMEDICAL GERONTOLOGY - IABG, BRIGHTON, UK, 11TH - 14 JULY 2011

DAN RIGA[1], SORIN RIGA[1], AUREL ARDELEAN[2], DANIELA MOTOC[2], FRANCISC SCHNEIDER[2]
[1]Department of Stress Research & Prophylaxis, Al. Obregia Clinical Hospital of Psychiatry, Bucharest, Romania
[2]Vasile Goldis Western University, Arad, Romania

ABSTRACT

The presentation, analysis and summary of the 61st Annual Scientific Meeting of the British Society for Research on Ageing – BSRA and of the 14th Congress of the International Association of Biomedical Gerontology – IABG are the alignment to the current stage and, especially, to future directions of transforming present day reality of an aging people with disabilities into long-lived, competitive psychologically and physically active human beings, with much longer ontogenetic periods. The focus on certain areas of research becomes essential for the speedier discovery and introduction of prophylactic, therapeutic and recovery solutions for human senescence and its transformation into human longevity.
Keywords: 61st Annual Scientific Meeting of the British Society for Research on Ageing - BSRA; 14th Congress of the International Association of Biomedical Gerontology - IABG; Science of aging; Biogerontology; Present period; Future directions.

MODEL OF MEETINGS FOR THE SCIENCE OF AGING

These **two international congresses** reunited under the title ***The Science of Ageing. Global Progress*** and connected meetings, held in Brighton, UK, 2011 (3, 10), represent a scientometric pattern to establish the present and future directions of a determinant scientific field for today - biogerontology. This science - the study of the biological basis of aging - has important significations in biomedical research, aging and health education, prophylaxis, therapy and recovery of senescence with age-related diseases, as well as in general and specific health care systems.

Adjacent events included:

- the BSRA Annual General Meeting and BSRA Executive Committee Meeting;

- Award of *the Lord Cohen Medal* to Prof. Dr. Suresh I. S. Rattan, at the Department of Molecular Biology, Aarhus University, Aarhus, DK, for his contribution to the science of gerontology. This medal is the highest honour the BSRA can bestow and its previous recipients include Robin Holliday, Leonard Hayflick and Tom Kirkwood;

- *Biogerontology,* Springer Editorial Board Meeting (Suresh I. S. Rattan, Editor-in Chief);

- Public Round Table Discussion by the Honorary Life Members of the BSRA to discuss *Physiological and ethical aspects of aging research* (Chair Prof. Alan Maryon-Davis; and Prof. Leonard Hayflick, Prof. Dame Linda Partridge, Prof. Tom Kirkwood, Prof. Arlan Richardson, Prof. Sydney Shall, Mrs. Elizabeth Mills);

- Funders Round Table Discussion - Chair Prof. R. Faragher and Representatives from *National Institute on Aging-National Institutes of Health* (NIA-NIH, USA), *Medical Research Council* (MRC, UK), *Biotechnology and Biological Sciences Research Council* (BBSRC, UK), *Australian Research Council* (ARC, AU), *Age UK* (the previously separate charities *Age Concern* and *Help the Aged,* which now together form the UK's largest charity for the elderly and *British Council - Research Council* (UK);

- NIA-BBSRC Joint Transatlantic Symposium and Workshop (Chair Prof. R. Faragher);

- PROTEOMAGE Session, UE (Chair Prof. Brian Clark);

- *American Aging Association* (AAA) Symposium (Chair Prof. Janko Nicolich-Zugich);

- *Chemistry Central Journal* Meeting for *Chemistry of Ageing* Special Issue (Section Editor Elisabeth Ostler).

Historical landmarks

Chair, Prof. Dr. Richard Faragher, University of Brighton, UK opened this local and international joint meeting emphasizing the importance of the ***British Society for Research on Ageing* - BSRA** (1, 5) and of the ***International Association of Biomedical Gerontology* - IABG** in the evolution and development of aging sciences as worldwide global progress. Also, historical landmarks are very significant because the BSRA was one of the pioneers of aging investigation, which grew out of the ***British Club for Research on Ageing*** founded by Dr. Vladimir

Received August 20th. Accepted September 21st. Address for correspondence: Sorin Riga, Department of Stress Research and Prophylaxis, "Al. Obregia" Clinical Hospital of Psychiatry, 10 Berceni Rd., 041914 Bucharest 8, Romania, Phone: +40 21 334 3008, Fax +40 21 230 95 79, e-mail: D_S_Riga@yahoo.com

Korenchevsky (1880-1959) in Oxford, in **1945,** and a leading organization in the creation of the International Association of Gerontological Societies in Liège, BE, in 1950 (5).

Congress themes

Up-to-date lectures, poster presentations and sessions, as well as interesting discussions focused on**:**

- the present and future **directions of aging research and biogerontology;**

- basic and fundamental **molecular and cellular mechanisms of aging processes;**

- immune system in aging, immune risk profile and experimental and medical immunological researches;

- Werner syndrome and its correlation with premature and accelerated aging;

- fundamental mechanisms of brain aging and its connection with neuropathology (as part of neurosciences), as well as **muscle aging mechanisms and interventions;**

- PROTEOMAGE session (Chair: Prof. Brian Clark);

- nutrition, nutrigenomics, calorie restriction mimetics; and

- exceptional longevity.

SENESCENCE INVESTIGATION: PRESENT AND FUTURE

The present and future **directions of aging research and biogerontology** was one of the main objectives of these two congresses:

- *The need for, and promise of, research on aging* (Elisabeth Mills, UK);

- *Developing knowledge management systems for aging research* (Alex Zhavoronkov, RU), a flexible open-access knowledge management system for aging research - the International Aging Research Portfolio (IARP) accessible via www.agingporfolio.org;

- *Aging - from here to where?* (Suresh Rattan, DK);

- *Health-longevity pyramid in the anti-aging global progress* (Sorin Riga, Dan Riga et al., RO), (9);

- *Ethical, social & cultural aspects of anti-aging science, biomedicine and older people* (Joanna Latimer, UK).

Elisabeth Mills, UK *(The need for, and promise of, research on aging)* pointed out ways to transform unsuccessful aging, a complex mixture of morbidity, social isolation, poverty and invisibility into a successful period of life. Research efforts to understand the biology of aging, which is the basis of senescence, should better coordinate with government and the third sector structures, measures and funds.

Alex Zhavoronkov, RU *(Developing knowledge management systems for aging research)* addressed a flexible open-access knowledge management system for aging research - the International Aging Research Portfolio (IARP) accessible via www.agingporfolio.org. The IARP system, highly modular and portable, may be used as a platform for developing other knowledge management systems for aging research.

Suresh Rattan, DK *(Aging - from here to where?)* presented the goal to extend health-span by elucidation and exploration of the successful and dynamic interactions among biological, psycho-social and environmental factors, because aging can be understood at various levels, from evolutionary and biological levels to psychological and sociological ones. At the molecular biological basal level, aging is characterized by the stochastic occurrence and progressive accumulation of molecular damages, with inefficiency and imperfection of the maintenance and repair systems, and finally failure of homeodynamics. The holistic view of senescence facilitates the setting up of a framework for understanding, researching and developing effective and realistic strategies for aging interventions.

Sorin Riga, Dan Riga et al., RO *(Health-longevity pyramid in the anti-aging global progress)* emphasized the construction of human health-longevity as a global and holistic progress, because man is a bio-psycho-social being. Consequently, future medicine will be and must be the medicine of health, mainly the planning of personalized and public health, together with the strategies of longevity, somatic and mental health (9).

Molecular and cellular mechanisms of senescence

Basic and fundamental **molecular and cellular mechanisms of aging processes** were largely presented and debated:

- *Cell senescence is a cause of aging* (Thomas von Zglinicki, UK);

- *Sources, transmission and effects of transcriptional noise in C. Elegans aging* (Hang Lu, USA, Quee Lim Ch'ng, UK);

- *Mechanism and functions of epigenetic reprogramming in mammalian development* (W. Reik, UK);

- *Epigenomics: understanding how nutrition modulates the aging process* (John Mathers, UK);

- *Maternal diet, epigenetics and programming of adult health* (Susan Ozanne, UK);

- *Induced pluripotent stem cells in aging research* (Alexandra Stolzing, DE);

- *Wine yeast as a novel model system to study chronological life span* (Augustin Aranda, SP);

- *Probing fundamental aging biology with model systems* (David Gems, UK);

- *Stress response profiles in aging human cells* (Dino Demirovic and Suresh Rattan, DK);

- *Application of cross species analysis of conserved pathways to the study of cellular aging* (João F. Passos et al., UK);

- *Identification of major biomarkers of aging* (Shiva Marthandan et al., DE);

- *A compartmentalized protein-protein interaction network as a novel model of aging* (Daniel V. Veres and Peter Csermely, HU).

Thomas von Zglinicki, UK *(Cell senescence is a cause of aging)* pointed out the adverse effects of cell senescence, mainly the pro-oxidant and inflammatory cellular phenotype, which is a delayed consequence of a DNA damage response (DDR),

dependent on signalling through p21 (CDKN1A), p38MAPK and (for the inflammatory phenotype) NF-kB. Adult peripheral and brain neurons also present a senescent phenotype, as characterized by DDR, p38MAPK activation, oxidative damage and interleukin production. nfkb1-/- mice show a constitutively activated inflammatory phenotype due to increased formation of p65 homodimers.

Hang Lu, USA, Quee Lim Ch'ng, UK *(Sources, transmission and effects of transcriptional noise in C. Elegans aging)* presented the beginning of a collaborative project with aims to determine how transcriptional noises in environmental responses translate to intoheterogeneity in lifespan and aging in nematode worm *C. Elegans* and to define environmental and genetic factors that contribute to these sources of noise. They have developed microfluidic and automated microscopy systems capable of collecting large scale data necessary for their analysis, as well as generating a molecular genetic pipeline for single copy insertions of transcriptional markers. In addition, they developed an array of devices for individual-animal long-term culture to assay aging phenotypes.

W. Reik et al., UK *(Mechanism and functions of epigenetic reprogramming in mammalian development)* spoke about epigenetic information in the genome, which is relatively stable in somatic cells, but is reprogrammed on a genome wide level in germ cells and early embryos. A key component of reprogramming is the erasure of DNA methylation, which may occur by passive (replication dependent) or active mechanisms. They used genome-wide profiling methods based on bisulfite sequencing (BS-Seq) or antibody pulldown sequencing (MeDIP-Seq) in order to understand better the dynamics of erasure of DNA methylation. The study of genome-wide BS-Seq in primordial germ cells has revealed the extent of reprogramming on an unprecedented scale, and has shown that the activated-induced cytidine deaminase (AID) is implicated in epigenetic reprogramming and potentially in active demethylation of DNA. Also, the distribution in the genome of methylation (5mC) and hydroxymethylation (5hmC) was investigated in (embryonic stem (ES) cells and during their differentiation, using MeDIP-Seq and hMeDIP-Seq. 5hmC is enriched in euchromatic parts of the genome, including in promoters of some pluripotency-related genes, where it is associated with transcription. There is a considerable turnover of methylation by hydroxymethylation in ES cells, which helps to maintain their plasticity and pluripotency.

João F. Passos et al., UK *(Application of cross species analysis of conserved pathways to the study of cellular aging)* developed the Cross-species Interactome Database (CID), an integrated resource for the analysis and comparison of interaction networks across different species. The CID database and the described computational methods are made available through a newly developed plugin for Cytoscape, an open source software platform for visualising and analysing molecular interaction networks.

Shiva Marthandan et al., DE *(Identification of major biomarkers of aging)* presented their research program, part of the JenAge project, with the aim to elucidate the molecular mechanisms and cellular pathways that drive cellular senescence in primary cell culture, mainly human fibroblasts.

Alexandra Stolzing, DE (iPS-*Induced pluripotent stem cells in aging research)* discussed about first iPs cell lines, recently created from donors with aging-accelerating genetic disorders (neurodegenerative disease or mitochondrial mutations), to elucidate the role of these mutations in aging processes.

David Gems, UK *(Probing fundamental aging biology with model systems)* in his closing keynote lecture of the congress addressed the accumulation of molecular damages in *C. Elegans* worm senescence as cause or not of the aging process.

Proteomage project

PROTEOMAGE Session (Chair: Prof. Brian Clark) presented this integrated EU-project (2006-2011), which used state-of-the-art proteomic technology to study molecular mechanisms of aging in various model organisms ranging from yeast to humans, as well as significant results with this topic:

- *From the stability of living cells to cellular stress-induced premature senescence and geriatric diseases* (Olivier Toussaint, BE);
- *Oxidative proteome alteration and protein maintenance during aging and upon oxidative stress* (Bertrand Friguet, FR);
- *The role of secreted proteins in cellular senescence and aging* (Pidder Jansen-Duerr, AT).

IMMUNE SYSTEM IN AGING

Immune system in aging, immune risk profile and experimental and medical immuno-logical researches were an important pillar of these traditional scientific meetings:

- *The immune system in aging* (Graham Pawelec, DE);
- *Steroid receptors and the control of thymic involution* (Nancy Manley, USA);
- *Mechanism of reduced T cell immunity in older adults* (Arne Akbar, USA);
- *Rejuvenation strategies to rebalance the aging T-cell repertoire* (Megan Smithey, USA);
- *A novel high-throughput autophagy assay for primary cells: senescent T cells show decreased levels of autophagy* (Kanchan Phadwal, UK);
- *Preparation of integral membrane proteins in order to evaluate cell surface protein expression in human CD4+ T-lymphocytes from young and mid-life adults* (S. J. Bennett et al. UK);
- *The role of peroxiredoxin antioxidant enzymes in stress resistance, aging and innate immunity* (E. L. Button et al., UK);
- *The effect of aging on regulatory B cells* (Niharika A. Duggal and Janet M. Lord, UK);
- *Immune system aging in heterochronic parabiosis: starting mechanism and impact on life expectancy* (I. Pishel et al., Ukraine);
- *Reversible senescence in human CD4+ CD45RA+CD27 - memory T cells* (D. Mitri et al., IT).
- *The impact of lifelong persistent viral infections on naïve and memory T-cell receptor Vb repertoire diversity* (P. Samadder,

USA);

- *Peripheral lymphocyte activation and differentiation in old melanoma patients* (J. A. Seidel, UK);

- *Impaired neutrophil extracellular trap (NET) formation: a novel defect in the innate immune system of aged individuals* (J. Hazeldine et al., UK);

- *Splenic architecture and cellularity is perturbed in aging mice and exhibit altered immune responses to T-independent and T-dependent antigens* (D. Aw et al., UK).

Graham Pawelec, DE *(The immune system in aging)* spoke about immune system in the elderly, which paradoxically suffer both from failing immunity resulting in increased susceptibility to infections, and decreased responsiveness to vaccination, and at the same time increased immunopathology accompanying immune responses. Limited longitudinal studies have begun to reveal biomarkers of immune aging increasingly recognized as an "immune risk profile" (IRP) predicting mortality in the very elderly. Usually asymptomatic infection with the widespread persistent β-herpesvirus HHV5 (Cytomegalovirus, CMV) has an enormous impact on these immune biomarkers. The prevalence of CMV infection in the population increases with age, and within individuals, the degree of immune commitment also increases with age. This may cause pathology by maintaining higher systemic levels of inflammatory mediators and decreasing the "immunological space" available for immune cells with other specificities.

Nancy Manley et al., USA *(Steroid receptors and the control of thymic involution)* emphasized the role of Androgen Receptor (AR) in postnatal involution of the thymus with age, and presented their initial findings on the expression and localization of AR in the thymus, the thymus phenotypes in $Ar^{Tfm/Y}$ mutant mice and on TEC-specific deletion of the AR.

Megan Smithey, USA *(Rejuvenation strategies to rebalance the aging T-cell repertoire)* covered the topic of immunosenescence in individuals with lifelong persistent viral infections, particularly with the Herpesvirus family members CMV and herpes simplex virus (HSV). In both mouse and human, repeated interactions between these reactivating viruses and antiviral T cells leads to memory T-cell inflation (MI), with increasing accumulation of these cells over life span. Her research team hypothesized and tested that MI carries a price for the immune system, namely competition between memory and naïve T-cells for survival signals may impair the maintenance of the diverse naïve T-cell pool, consequently leaving the individual at a disadvantage when exposed to a new pathogen.

D. Aw et al., UK presented *Splenic architecture and cellularity is perturbed in aging mice and exhibit altered immune responses to T-independent and T-dependent antigens.* Indeed, these age-related changes within the spleen could potentially contribute to the age-dependent deficiencies in functional immunity.

CEREBRAL AGING

Fundamental mechanisms of **brain aging and its connection with neuropathology** (as part of neurosciences) are presented at length:

- *A RNA-seq analysis of brain aging in the Brown rat* (Shona Wood, UK);

- *A senescence-like phenotype in postmitotic neurons* (C. Wang et al., UK);

- *How well do sleep and activity behaviours predict lifespan in Drosophila?* (S. Koudounas et al., UK);

- *Upregulation of the synaptic plasticity regulating major histocompatibility complex I pathway in the hippocampus with aging and cognitive decline* (C. Van Kirk et al., USA);

- *A new Caenorhabditis elegans model of age-dependent neurodegeneration* (Sudhanva Kashyap, UK);

- *A complex dietary supplement prevents age-related cognitive and motor declines and offsets cellular biomarkers of aging in mice* (Vadim Aksenov, CA);

- *Supplementation of tocotrienol rich fraction (TRF) improved cognitive functions in aged rats* (M. T. Nursiati et al., Malaysia);

- *Perceptual plasticity in the peripheral visual field of older adults* (A. Blighe et al., UK);

- *Senile local eye amyloidosis risk factor for age-related macular degeneration* (V. V. Ermilov and O. V. Makhonina, RU);

- *Upregulation of calpastatin may protect motor neuron death during the development amyothrophic lateral sclerosis* (Liang Li and Adu Jimi, UK);

- *Pharmacological modulation of the abnormal oscillatory profile in Parkinson's disease* (K. Ronnqvist et al., UK);

- *Aβ and oxidative stress in the brain of the longest-living rodents* (Y. Edrey et al., USA);

- *Brain lipopigments - link between aging and Alzheimer disease* (Dan Riga, Sorin Riga et al., RO), (8);

- *Saffron (Crocus sativus L.) extract prevents and improves the Alzheimer's disease in mice* (M. H. Dashti et al., Iran);

Shona Wood and co-workers, UK *(A RNA-seq analysis of brain aging in the Brown rat)* used RNA-seq examination (a more accurate method than mRNA microarrays) to sequence the cerebral cortex transcriptome in young, middle age and old rats. This analysis allows non-coding RNA and splice variant detection/comparison across phenotypes. The study showed that there is a relatively large amount of non-coding RNAs differentially expressed with aging in the cerebral cortex. They have a regulatory role in gene expression and splice variant selection.

C. Wang et al., UK *(A senescence-like phenotype in postmitotic neurons)* presented (for the first time) that DNA damage triggers a senescent-like phenotype, characterized by activation of p38 MAPK, production of reactive oxygen species (ROS) and pro-inflammatory cytokines and increased levels of senescent-associated β-galactosidase in brain and peripheral neurons.

Sudhanva Kashyap and collab., UK *(A new Caenorhabditis elegans model of age-dependent neurodegeneration)* described this type of worm produced by mutation of the *dnj-14* gene. DNJ-14 is the worm homologue of cysteine string protein (CSP), a neuronal protein that prevents the misfolding of presynaptic proteins. Besides, CSP knockout mice have a short lifespan

and exhibit progressive age-related neurotransmission defects, sensiomotor dysfunction and pre-synaptic neurodegeneration.

Y.H. Edrey et al., USA *(Aβ and oxidative stress in the brain of the longest-living rodents)* used Naked Mole-Rats (NMRs, *Heterocephalus glaber*), subterranean rodents, evolved in a hypoxic habitat, naturally vitamin D deficient, with a maximum lifespan of 32 yrs. In captivity they incur high levels of oxidative damage evident even at a young age. Their sequence of Aβ peptide was more similar to the human peptide, than to that of its mouse counterpart. NMRs brains had a detectable amount of both soluble and insoluble fractions of Aβ, even at a young age and showed significantly higher levels than young adult humans or transgenic mice that express human Aβ.

Dan Riga, Sorin Riga and co-workers, RO *(Brain lipopigments - link between aging and Alzheimer disease)* studied cerebral lipopigments - LPs (lipofuscin and ceroid) in normal aging in comparison with Alzheimer disease (AD). In AD, the LPs become a constancy and are associated with specific neurodegenerative changes. Moreover, LP storages are constitutive parts of amyloid (senile, neuritic, argyrophilic) plaques and neurofibrillary tangles (8).

SENESCENCE OF MUSCULAR SYSTEM

The **Muscle aging** session, with constant lectures at every biogerontology meeting, presented the mechanisms of sarcopenia and interventions to maintain muscle mass and function:

- *Can exercise/physical activity prevent loss of motor units?* (Susan Brooks, USA);

- *Aging, inflammation, and weakness* (Mike Reid, USA);

- *Do oxidative damage or defective redox signalling contribute to age-related loss of skeletal muscle?* (Malcolm Jackson, UK);

- *Changes in muscle protein homeostasis with aging* (Marco Sandri, IT);

- *Overexpression of HSP10 in transgenic mice protects skeletal muscle against endotoxic shock-induced loss of force generation* (Anna Kayani et al., UK);

- *Insulin/IGF-like signalling and aging-related locomotor decline in Drosophila melanogaster* (Sue Broughton, UK);

- *Losartan restores skeletal muscle remodeling and protects against disuse atrophy in sarcopenia* (T. N. Burks et al., USA);

- *Life events stress and physical activity are associated with the diurnal rhythm of dehydroepiandrosterone and the cortisol: DHEA ratio in older adults* (J. Heaney et al., UK);

- *Metabolic equivalent of a one-mile walk by older adults: implications for health promotion* (M. Gault et al., UK);

- *ROS generation during myogenesis: role of altered ROS generation in failed regeneration of old muscle* (A. Vasilaki et al., UK).

In *Aging, inflammation, and weakness,* **Mike Reid, USA** presented correlations between these concepts. As such, ceramide levels and sphingomyelinase activity are elevated in senescence, and also depress specific force. Cytosolic oxidants preferentially

act on myofilrillary proteins, decreasing specific force, without altering calcium regulation or other aspects of myofibrillar mechanics.

Marco Sandri, IT *(Changes in muscle protein homeostasis with aging)* emphasized his recent data that autophagy-lysosome system is critical to maintain muscle mass, and its alteration leads to muscle atrophy, weakness and to several features that are present in aging sarcopenia.

T. N. Burks et al., USA *(Losartan restores skeletal muscle remodeling and protects against disuse atrophy in sarcopenia)* studied the increased TGF-β signalling, which contributes to impaired satellite cell function and muscle repair in aged skeletal muscle. They evaluated whether antagonism of TGF-β signalling via losartan, an angiotensin II receptor antagonist commonly used to treat high blood pressure, had a beneficial impact on the muscle remodelling process of sarcopenic mice. They demonstrated that mice treated with losartan developed significantly less fibrotic tissue and exhibited improved *in vivo* muscle function after cardiotoxin-induced injury.

Efficient therapies and strategies to preserve mobility and movements in the elderly become very useful.

WERNER SYNDROME - WS

Werner syndrome - WS (described by Otto C. W. Werner, 1879-1836, German physician) in the young adult (child's equivalent disease - Hutchinson-Gilford, individualized by Jonathan Hutchinson, 1828-1913, English surgeon and Hastings Gilford, 1861-1941, English physician) was debated in **its connexion with premature and accelerated aging:**

- *Towards a cure for adult progeria* (David Kipling et. al., UK);

- *Using phage display to screen for protein partners of WRN, implicated in premature aging Werner syndrome* (Lynne S. Cox and Ivan Boubriak, UK);

- *Assessing the role of p38 MAP kinase activity in replicative senescence in fibroblasts from progeroid syndromes* (Terence Davis et al., UK);

- *Exploring the function of progeroid WRN gene homologues in Caenorhabditis elegans* (H. Lees et al., UK).

David Kipling et. al., UK *(Towards a cure for adult progeria)* focused their research on p38 activation in WS, which upregulate immunomodulatory molecules such as pro-inflammatory cytokines. The premature aging in WS may be explained exclusively by the accumulation of senescent cells in certain lineages, exclusively as a result of p38-driven "inflamm-aging", or a combination of both. p38 inhibitors are being actively developed by pharmaceutical groups, raising opportunities to test potential therapeutic approaches *in vivo.*

Lynne S. Cox and Ivan Boubriak, UK *(Using phage display to screen for protein partners of WRN, implicated in premature aging Werner syndrome)* insisted on WS as premature human aging model. WS is result from loss of functional WRN, a large protein with multiple active domains including a DNA helicase and a 3´-5´exonuclease. This protein is implicated in many aspects of DNA metabolism comprising DNA replication, DNA repair and homologous recombination. They recently developed a fly model

in which the exonuclease component of WRN, DmWRNexo is expressed at extremely low levels. Such flies show WS-like phenotypes of sensitivity to the topoisomerase poison camptothecin, and very high rates of DNA recombination. Patients with WS are essentially null for both the helicase and exonuclease activities of WRN, and show elevated rates of DNA recombination.

H. Lees et al., UK *(Exploring the function of progeroid WRN gene homologues in Caenorhabditis elegans)* discussed the correlation between the WRN human gene, encoding both helicase and exonuclease activities and progeroid WRN gene homologs in *C. Elegans.* Their comparative genomic analyses in *C. Elegans* suggested that *wrn-1* is the closest homologue of the human WRN helicase domain, and *mut-7* is the closest homologue of the human WRN exonuclease domain. Therefore, this worm model can be used to dissect at the molecular level the relative impact of the WRN helicase and exonuclease to DNA damage responses and genome stability.

EXCEPTIONAL LONGEVITY

Exceptional longevity, in animals and humans, represents an important concept and tool for understanding aging mechanisms. Therefore, this subject was present at each biogerontology meeting:

- *Mechanisms of exceptional longevity in the world's longest lived animal* (Iain Ridgway, USA);
- *Hearty healthspan and lengthened lifespan: the contribution of cytoprotection in the extraordinarily long-lived naked mole-rat* (Kaitlyn Lewis, USA);
- *Proteome stability and exceptional longevity in bivalve molluscs* (S. Treaster, USA);
- *Transcriptome analysis of the long lived bivalve Artica islandica* (E. Philipp, DE);
- *Extreme life histories: the long-lived ocean quahog Artica islandica* (R. Schaible, DE);
- *Comparing gene expression between octo/nonagenarians and offspring to elucidate biological processes involved in aging* (A. Abdul Rahman, Malaysia);
- *Antioxidant profiles do not explain the decreased susceptibility to oxidative stress in Malaysian octo/nonagenarians* (Yasmin Anum Mohd Yusof, Malaysia).

Iain Ridgway and co-workers, USA, UK and DE *(Mechanisms of exceptional longevity in the world's longest lived animal)* investigated the uncommon longevity exhibited by the ocean aquatic molluscs (Phylum *Mollusca*) of the class *Bivalvia* (long-lived *Artica islandica,* the world's longest living non-colonial animal, in comparison with the shorter-lived *Mercenaria mercenaria).* First, they comparatively investigated biochemical profiles, age related change responses to exposure to the oxidative stressor Tert-Butyl HydroPeroxide (TBHP). Following TBHP exposure, an association between longevity and resistance to oxidative stress-induced mortality, and also a marked resistance to oxidative stress-induced cell death was observed in *A. islandica* compared with *M. mercenaria*. Second, they have developed new approaches for investigation of proteome stability, which included assessment of cytosolic carbonyls, total insoluble carbonyls, insoluble carbonyls localized to either nuclear, microsomal or mitochondrial fractions, and protein structural stability in the face of unfolding stress. The pattern of proteome homeostasis and stability are different between long-lived and short-lived species.

In addition, very promising research performed by **E. Philipp, R. Schaible et al., German team from Kiel and Rostock, DE** *(Extreme life histories: the long-lived ocean quahog Artica islandica* and *Transcriptome analysis of the long lived bivalve Artica islandica),* identified extreme differences in lifespan among *Artica islandica* populations: short-lived *Baltic Sea population,* with max. lifespan < 50yrs., and extremely long-lived *Icelandic population,* with max. lifespan of > 400 yrs.

Kaitlyn Lewis, USA *(Hearty healthspan and lengthened lifespan: the contribution of cytoprotection in the extraordinarily long-lived naked mole-rat)* described investigations on naked mole rat *(Heterocephalus glaber,* Rüppel, 1842), a burrowing rodent native to parts of East Africa, which holds the record for the longest living rodent, up to 32 yrs. (maximum lifespan). There is a positive correlation between the transcription factor Nrf2 (nuclear factor-erythroid 2-releated factor-2) signalling activity and species longevity. Upregulation of the Nrf2- signalling pathway may be a conserved mechanism contributing to hearty healthspan and lengthened lifespan. It is important to mention that Nrf2 regulates the transcription of over 200 cytoprotective genes with key roles in protection against aging, toxins, inflammation, neurodegeneration and cancer, and is also implicated in aging and longevity.

NUTRITION IN AGING

Nutrition, nutrigenomics, calorie restriction mimetics also pointed out the importance in ontogenesis of food normality and physiological good lifestyles:

- *Maternal diet affects the aging trajectory* (S. Barnes, UK);
- *The influence of early nutrition on splenic aging* (C. Heppolette, UK);
- *Does atypical methionine metabolism contribute to longevity?* (Holly Brown Borg, USA);
- *Depletion of mTOR and mLST8 uncouples longevity from rapamycin-induced changes in glucose homeostasis* (Dudley W. Lamming, UK);
- *Is rapamycin an anti-aging drug?* (Arlan Richardson, USA);
- *Caloric restriction mimetics: state of flux* (Don Ingram, USA).

John Mathers, UK *(Epigenomics: understanding how nutrition modulates the aging process)* presented two separate approaches to investigate nutrition mechanisms influence and the biological processes responsible for the senescence phenotype. For the discovery of genes the expression of which is epigenetically regulated and which are susceptible to modification by dietary factors he used a combination of genome-wide analysis of gene expression and of genome-wide methylation of CpG islands and gene promoters. Moreover, he studied nutritional

modulation of brain aging, focused on DNA mechanisms in a mouse model exposed to nutritional insults in early life.

Susan Ozanne and collab., UK *(Maternal diet, epigenetics and programming of adult health)* demonstrated within the framework of "early life programming" concept how maternal diet can mediate its effects on subsequent age-associated diseases. Their findings pointed out that expression of HNF4 alpha, a key developmental transcription factor previously implicated in the risk of diabetes, was reduced in pancreatic islets following suboptimal nutrition in early life through epigenetic mechanisms.

S. Barnes et al., UK *(Maternal diet affects the aging trajectory. A microarray approach)*

emphasized that maternal diet modulates age-associated changes in renal gene expression, that may contribute to its effects on kidney function and ultimately lifespan.

C. Heppolette and co-workers, UK *(The influence of early nutrition on splenic aging)* through their research argue the Developmental Origin of Health and Disease Hypothesis, which proposes that environmental factors such as nutrition during early life influence long-term health. The spleen, the major secondary lymph organ and site of peripheral antigen recognition, was investigated. They used the low protein mouse model of nutritionally-induced foetal growth restriction and postnatal catch-up growth (recuperated) which results in reduced lifespan, and demonstrated that low birth weight and catch-up growth leads to accelerated aging of the spleen.

Arlan Richardson and collab., USA *(Is rapamycin an anti-aging drug?)* brought out the effective anti-aging effects of rapamycin, an FDA approved drug for human use for tissue transplant therapy and treating certain types of cancer. Using two transgenic mouse models of Alzheimer disease (AD), they showed that feeding mice rapamycin at a level that increased lifespan rescued cognitive deficits and ameliorated Aβ and tau pathology, by autophagy. In *ApoE*$^{-/-}$ mice fed a high fat diet, feeding rapamycin attenuated the development and progression of atherosclerotic lesions. In addition, feeding rapamycin prevented the development of colon cancer in a transgenic model (Apc$^{Min/+}$) of adenomatous polyposis.

Don Ingram, USA *(Caloric restriction mimetics: state of flux)* emphasized the importance of Calorie Restriction (CR) for anti-aging and health-promoting benefits, practically the most robust means to retard aging in numerous species. But, despite its strong beneficial effects in humans, it is difficult to apply and to be accepted by individuals, due to their nutritional habits and wrong lifestyles. Therefore, the CR mimetics (CRM) has been introduced to obtain the same positive effects, without reducing food intake. Several candidate CRM compounds have been proposed (sirtuin activator, resveratrol, metformin, rapamycin), but only the latter has produced consistent prolongevity effects in mice with normal diet.

CONTINUITY OF THE IABG CONGRESSES

The International Association of Biomedical Gerontology (IABG) was founded by Denham Harman in 1985. We must mention the importance of previous IABG congresses, where Prof. Denham Harman (originator of the free radical theory of aging and disease) was very active, as communicator, session chairman, organizer, and editor (4, 12).

In addition, the 10th Congress of IABG, held on September 19-23, 2003 in Cambridge, United Kingdom (7), was the hallmark in the conceptual evolution and development of bio-gerontology, aging control and prolongevity. The meeting papers were published as volume, Editor Aubrey D. N. J. de Grey, *Strategies for Engineered Negligible Senescence - SENS. Why Genuine Control of Aging May Be Foreseeable,* Annals of the New York Academy of Sciences, vol. 1019, New York Academy of Sciences, New York, NY, June 2004 (2).

Practically, the next IABG congresses - Aarhus, DK, 2005 (6); Spetses Island, GR, 2007; Quebec, CA, 2009 (11); and this in Brighton, UK, 2011 (3) - had the same topics and directions, but up-to-date and with relevance to new projects, search groups and consortia of aging investigation and rejuvenation research.

As previous scientific meetings, these two congresses from Brighton 2011 were models for integration of aging sciences: from genomics, proteomics, transcriptomics to cytomics and epigenomics, and from fundamental studies to translational medicine and clinical applications.

REFERENCES

1. Bowman C, Dubbey R, Ford P et al: *Standards of medical care for older people. Expectations and recommendation.* London, UK, British Geriatric Society, 1997.
2. de Grey ADNJ (ed): *Strategies for Engineered Negligible Senescence - SENS. Why Genuine Control of Aging May Be Foreseeable.* Annals of the New York Academy of Sciences, vol. 1019. New York, NY, New York Academy of Sciences, 2004.
3. Faragher R: *The Science of Ageing. Global progress.* Brighton, UK, University of Brighton, 2011.
4. Harman D, Holliday R, Meydani M (eds): *Towards Prolongation of the Healthy Life Span. Practical Approaches to Intervention.* Annals of the New York Academy of Sciences, vol. 854. New York, NY, New York Academy of Sciences, 1998.
5. Phillips J (ed): *British gerontology and geriatrics: experience and innovation.* London, UK, British Geriatric Society, 1997.
6. Rattan SIS, Kristensen P, Clark BFC (eds): *Undestanding and Modulating Aging.* Annals of the New York Academy of Sciences, vol. 1067. Boston, MA, Blackwell 2006.
7. Riga D: SENS acquires SENSe: present and future anti-aging strategies, 10th Congress IABG - International Association of Biomedical Gerontology, Queens' College, Cambridge, UK, September 19-23, 2003. *Journal of Anti-Aging Medicine* 2003; 6(3): 231-236.
8. Riga D, Riga S, Ardelean A, Pribac G, Motoc D, Schneider F: Brain lipopigments - link between aging and Alzheimer disease, *61st Annual Scientific Meeting of the British Society for Research on Ageing - BSRA & 14th Congress of the International Association of Biomedical Gerontology - IABG,* Brighton, UK, 11th - 14 July 2011.
9. Riga S, Riga D, Ghinescu M, Mihăilescu A, Motoc D, Geacăr S: Health-longevity pyramid in the anti-aging global progress. *61st Annual Scientific Meeting of the British Society for Research on Ageing - BSRA & 14th Congress of the International Association of Biomedical Gerontology - IABG,* Brighton, UK, 11th - 14 July 2011.
10. Riga D, Riga S: The science of aging: global progress, *Rejuvenation Research* 2011; 14(5): in press.

11. Tanguay RM (ed): *Aging, Cancer, and Age-Related Diseases*. Annals of the New York Academy of Sciences, vol. 1197. Boston, MA, Blackwell, 2010.
12. Zs.-Nagy I, Harman D, Kitani K (eds): *Pharmacology of Aging Processes. Methods of*
13. *Assessment and Potential Interventions.* Annals of the New York Academy of Sciences, vol. 717. New York, NY, New York Academy of Sciences, 1994.

BIOGERONTOLOGIA ASTAZI. PARTEA I. A 61-A CONFERINȚĂ ȘTIINȚIFICĂ ANUALĂ A SOCIETĂȚII ENGLEZE DE CERCETARE A ÎMBĂTRÂNIRII - BSRA & AL 14-LEA CONGRES AL ASOCIAȚIEI INTERNAȚIONALE DE GERONTOLOGIE BIOMEDICALĂ - IABG, BRIGHTON, ANGLIA, 11 - 14 IULIE 2011

REZUMAT

Prezentarea, analiza şi sinteza celei de a 61a Sesiune Ştiinţifică Anuală a Societăţii Britanice pentru Cercetarea Îmbătrânirii - BRSA şi celui de al 14lea Congres al Asociaţiei Internaţionale de Gerontologie Biomedicală - IABG reprezintă înscrierea stadiului prezent cât, mai ales, a direcţiilor viitoare de a transforma umanitatea în curs de îmbătrânire cu dizabilităţi, în fiinţe umane longevive cu o sănătate optimă, competitivă şi active fizic şi psihic perioade ontogenetice mult mai lungi. Concentrarea pe anumite direcţii de cercetare devine esenţială pentru găsirea şi introducerea mai rapidă a soluţiilor profilactice, terapeutice şi de recuperare în senescenţa umană şi trensformarea ei în longevitate umană.
Cuvinte cheie: A 61a Sesiune Ştiinţifică Anuală a Societăţii Britanice pentru Cercetarea Îmbătrânirii - BRSA; Al 14lea Congres al Asociaţiei Internaţionale de Gerontologie Biomedicală - IABG; Ştiinţa îmbătrânirii; Biogerontologie; Stadiul prezent; Direcţii viitoare.

EVALUATION OF SKIN PARAMETERS CHANGES IN CHEMICAL AND PHOTOCHEMICAL INITIATED TUMORS ON SKH1 MICE

SORINA CIURLEA[1], MARIA-FLORINA BOJIN[2], ERZEBET CSANYI[3], DANIELA IONESCU[1], FLORIN BORCAN[4], ATENA GALUSCAN[5], CRISTINA A. DEHELEAN[1*]

[1] University of Medicine and Pharmacy "Victor Babes" Timisoara, Faculty of Pharmacy, Toxicology Dept., 2nd Eftimie Murgu Sq., Timisoara-300041, Romania

[2] University of Medicine and Pharmacy "Victor Babes" Timisoara, Faculty of Medicine, Physiology Dept., 2nd Eftimie Murgu Sq., Timisoara-300041, Romania

[3]University of Szeged, Faculty of Pharmacy, Pharmaceutical Technology Dept. Szeged, Hungary

[4] University of Medicine and Pharmacy "Victor Babes" Timisoara, Faculty of Medicine, Biochemistry Dept., 2nd Eftimie Murgu Sq., Timisoara-300041, Romania

[5] University of Medicine and Pharmacy "Victor Babes" Timisoara, Faculty of Dentistry, 2nd Eftimie Murgu Sq., Timisoara-300041, Romania

ABSTRACT

Ultraviolet B (UVB) or medium wave irradiation, with a wavelength in the range 315-280 nm and a energy per photon between 3.94-4.43 eV, regulates ultraviolet-responsive genes, including matrix metalloproteinases. Moreover, it causes connective tissue damage and the skin to become wrinkled and aged. The aim of this study was to investigate the evolution of the mouse skin papillomas initiated by 7,12-dimethylbenzanthracene (DMBA) and promoted by 12-O-decanoylphorbol-13-acetate (TPA), which progressed more rapidly to carcinoma on mice exposed to UVB. The biological effects of these two tumors (chemically and photochemically initiated) were evaluated for twelve weeks on the SKH1 hairless mice skin by using of non-invasive techniques (tewameter, skin-pH-meter, melanin and erythema measurements). It was found that UVB irradiation affects very much the parameters which were monitored and which characterizing the skin condition.

Keywords: tumor, DMBA, TPA, UVB, SKH1 mouse, non-invasive technique

INTRODUCTION

Skin cancer, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most common malignant neoplasms in humans (1,2). It has been estimated that cases of BCC and SCC are diagnosed each year and are increasing dramatically. Also this is equivalent with the incidence of malignancies in all other organs combined (3,4).

Chronic exposure to ultraviolet (UV) radiation is responsible for about 90% of human skin cancers and is a cancer promotor (5). Solar UV radiation is conventionally divided into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). The UV light that reaches the earth is composed of about 90% UVA and 10% UVB (6). Both UVA and UVB are being studied for their skin cancer-causing potential, but currently UVB is thought to be the most important etiologic factor and UVB is the most frequently used photocarcinogen in animal studies (7, 8)

Hairless mice are valuable for experimental carcinogenesis and more specific skin carcinogenesis studies. Carcinogens as initiators or promoters, chemopreventive agents, and chemotherapeutic compounds are readily applied to unperturbed hairless skin (9). SKH1 mice are highly susceptible to UVR-induced skin cancer (10) this susceptibility may be a function of the hairless gene itself, as C3H hairless mice. They are more susceptible to UVR carcinogenesis than haired mice even it is the same strain (11). A classification scheme for UVR-induced skin tumors in SKH1 mice accurately discriminates multiple stages in the carcinogenic stages of evolution (12).

There are some reports that suggest that 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes 7,12-dimethylbenz[a] anthracene (DMBA)-initiated mouse skin carcinogenesis, which is closely related to the inflammatory responses and also to carcinoma.

In the present study we examined the tumor-promoting activity of UVR, DMBA and TPA-induced acute inflammation in a multi-stage mouse skin carcinogenesis model, an ideal system to study a number of biochemical alterations in the different stages of chemical carcinogenesis (13).

MATERIALS AND METHODS

Animals

SKH1 mice of eight weeks were purchased from Charles River (Sulzfeld, Germany), female, 8 weeks age. The work protocol followed all NIAH - National Institute of Animal Health rules: animals were maintained during the experiment in standard conditions offered by the Biobase of University of Medicine and Pharmacy Timisoara: 12 hours light-dark cycle, food and water

Received June 14th 2011. Accepted August 15th 2011. Address for correspondence: Cristina-Adriana DEHELEAN, Department of Toxicology, Faculty of Pharmacy, University of Medicine and Pharmacy "Victor Babes" Timisoara, 2nd, E. Murgu Sq. 300041 Timisoara, RO, E-mail: cadehelean@umft.ro

ad libidum, temperature 24 °C, humidity above 55%. 11 mice were taken into study and were divided in three groups (three for blank and four for each tumor model). It was obtained the permission from the Ethical Commission on Animal Experiments of the University of Medicine and Pharmacy Timisoara.

Chemical treatment

7,12-Dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma (Germany). Mice were topically treated once in the first experimental week with DMBA solution (25 nmol in 200 µl acetone) and in the following weeks the DMBA was replaced by TPA solution, twice weekly (6.8 nmol in 200 µl acetone)(14).

UVB exposure

For UVB exposure, cages were placed in an automatically time-switched irradiation setup. For UVB exposure, cages were placed in an automatically time-switched irradiation setup. In the experiment, VL-6.M/6W (312 nm wavelength and 680 µW/cm^2 intensity at 15 cm) tubes (Vilber Lourmat, France) were used. Under the lamps the minimal erythema dose (MED) of hairless SKH-1 mice, was ≈300 J/m^2. The irradiation was twice weekly, 5 minutes/day, 2 times/week, hairless SKH-1 mice were exposed to a total dose arround 200 J/m2 UVB radiation.

Characterization of skin parameters

Each skin parameter determination was performed before any application or UVB exposure. All the measurements on the mice skin were carried out with a Multiprobe Adapter System (MPA5) from Courage&Khazaka Electronics, Germany, equipped with a Tewameter® TM300 probe for measurements of transepidermal waterloss (TEWL), a Skin-pH-meter® PH905 probe and a Mexameter® MX18 probe for determinations of melanin and erythema.

Statistics

Data are presented as mean + SEM and were analysed using one-way ANOVA and Tukey's post hoc analysis.

RESULTS

In vivo models like mouse or rats are important and highly adapted methods for studying cancer development and treatment surveillance and easy to reproduce (14, 15). The models that included chemical initiators and promoters like polycyclic aromatic hydrocarbures and phorbol esters ± other factors like UVB are frequent use for studying skin carcinoma and melanoma development. The two models of skin tumor initiation, a single application of the chemical initiator mutagen 7,12-dimethylbenz[*a*] anthracene (DMBA), followed by repeated applications of a pro-inflammatory phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) is an easy adaptable one. It can be applied on SKH1 mice also with skin tumor development (16, 17). The results are also easy observable (Fig 1).

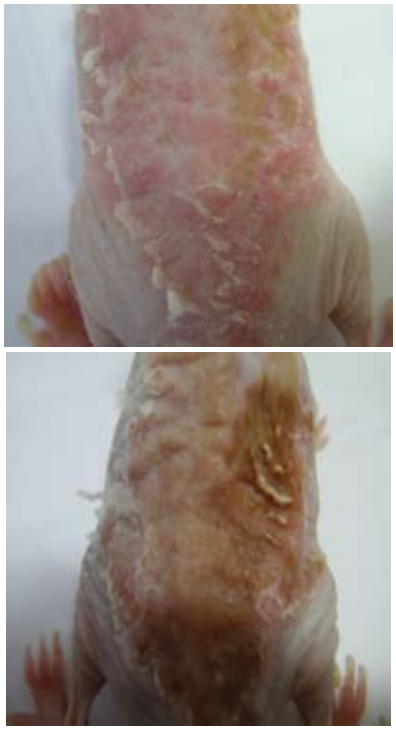

Fig.1. The mascroscopic images of SKH1 mice on week 12 of experiment A. application of DMBA/TPA and B. Application of DMBA, TPA and UVB radiation

The non-invasive methods can offer quantitative data and the image of pathology evolution in experimental models (Fig 2, 3, 4, 5).

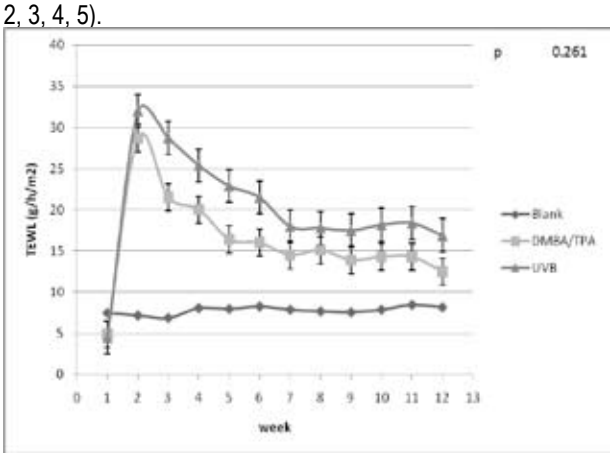

Fig. 2. The TEWL evaluation for UVB group comparing to group treated with carcinogens (DMBA/TPA) and blank group
(data are expressed as mean ± SEM, *p* is UVB vs. DMBA/TPA)

The TEWL measurements indicate a very large increase of transepidermal waterloss after the first week of applications. It is well-known that values of the transepidermal water loss under 10 g/h/m2 are characteristic of a skin in good condition (see the blank group from fig 2) and over 25 g/h/m2 correspond to a skin in bad and very bad condition. So, the application of DMBA in this week changed a lot the skin condition. The loss in the coming weeks is not so pronounced and the skin condition begins to recover.

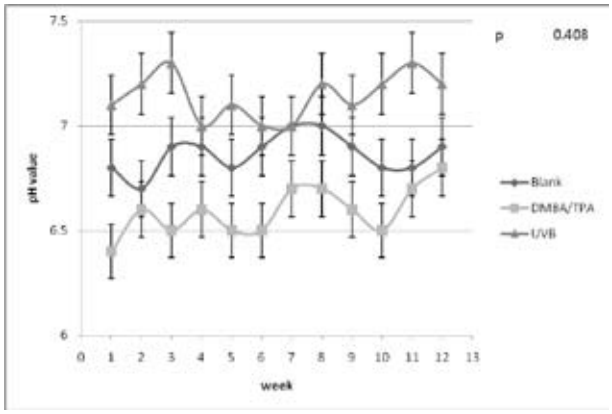

Fig. 3. The skin-pH evaluation for UVB group comparing to group treated with carcinogens (DMBA/TPA) and blank group (data are expressed as mean ± SEM, *p* is UVB vs. DMBA/TPA)

The pH evolutions are chaotics. The big values for standard errors of the mean and *p* are sufficient grounds to consider that these tests on the SKH1 mice skin does not affect the their skin-pH values (Fig 3).

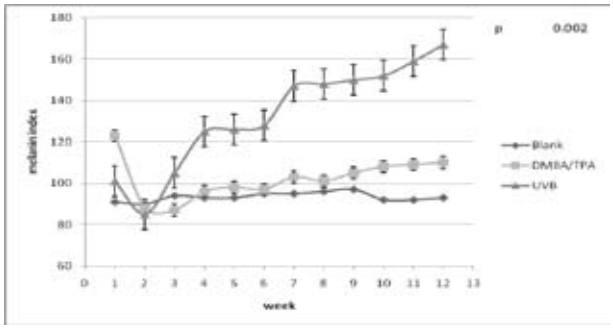

Fig. 4. The melanin skin content evaluation for UVB group comparing to group treated with carcinogens (DMBA/TPA) and blank group (data are expressed as mean ± SEM, *p* is UVB vs. DMBA/TPA)

The melanin measurements indicate a very large decrease, for the groups with carcinogens and UVB exposure, in the second week which is an extra argument to the TEWL modification due to the toxicological DMBA effect.

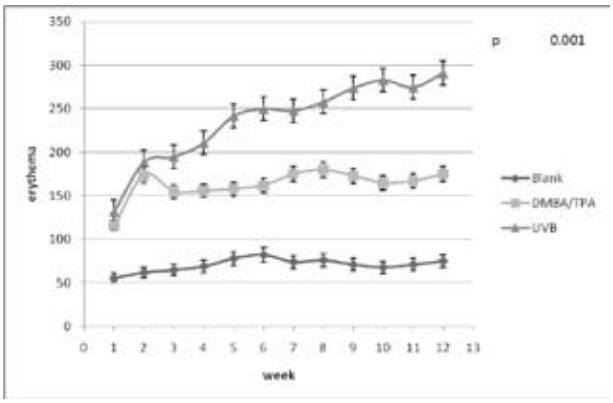

Fig. 5. The erythema evaluation for UVB group comparing to group treated with carcinogens (DMBA/TPA) and blank group (data are expressed as mean ± SEM, *p* is UVB vs. DMBA/TPA)

The evolutions of erythema average values show the very toxicological effect of DMBA and the aggressive effect of UVB on the mice skin. The UVB group record a continued increase during the twelve weeks.

DISCUSSIONS

Over-exposure to UV from the sun can cause sunburn, skin damage and in the final steps skin cancer. UVB can act in mouse skin models as a complete carcinogen, meaning that UVB can function as an initiator as well as a promoter (18, 19) and for this reason the combination of chemical carcinogens with UVB determine intense skin damages. Tumors and skin damages are easily identified and observable (Fig1). Each mouse develops multiple independent skin tumors, which may exhibit significant differences in rate of development and aggressiveness. Skin tumors progress from area of epithelial hyperplasia to papillomas and ultimately into squamous cell and spindle cell carcinomas and the evolution is in weeks (9). In first 12 weeks lesions, skin physiology and inflammation seems to be a general characteristic.

The SKH1 hairless mouse develops lesions resembling UVR-induced tumors and it is widely considered to be the most suitable mouse model for studies of UVR carcinogenesis (20). Our study confirms this general aspect.

The first responses after carcinogens application include the development of edema, hyperplasia, induction of pro-inflammatory cytokine interleukin-1a, enhanced release of reactive oxygen species, induction of epidermal ornithine decarboxylase (ODC) and over-expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) proteins and are related to initial step of a carcinogenic process (21, 22). Increased expression of COX-2 and/or iNOS was observed in many different types of tumors and transformed cells (23, 24). Besides its ability to act as an initiator of carcinogenesis, nitric oxide is also involved in the promotional stage of tumorigenesis (25) and in tumor progression by regulating angiogenesis (26).

Tumors are graded as papillomas microinvasive squamous cell carcinomas or fully invasive SCCs (12). This aspect is observable by transepidermal waterloos and also by erythemal evolution. The other parameters confirm the evolution of skin pathology.

These measurements indicated important changes from the first weeks of experiments and also the most increased process after 10 weeks. The erythemal values and changes in skin water content are obvious more increased in case of the UVB adding. Melanin is not very much influenced in case of SKH1 mice on the first weeks of experiment.

CONCLUSIONS

Experimental models on sensitive specific animals are applicable in cancer research and also in models like skin carcinoma. Increasing the number of carcinogenic factors, such as chemicals and UV radiation, leads to a faster evolution of pathology and to important skin damages. The detailed observation of these aspects can be performed by application

of non-invasive methods that establish important changes in skin physiology during pathology evolution. After a few weeks of exposure to initiators and promoters mouse skin suffer important changes and the major damages are in a started process.

REFERENCES

1. Dehelean CA. *Toxicologie. Notiuni generale de toxicologie*. Ed. Mirton, Timisoara, 2008: 7-16.
2. Mantena SK, Sharma SD, Katiyar SK. Berberine inhibits growth, induces G1 arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating Cdki-Cdk-cyclin cascade, disruption of mitochondrial membrane potential and cleavage of caspase 3 and PARP. *Carcinogenesis* 2006; 27:2018-2027.
3. Naylor MF, Boyd A, Smith DW, et al. High sunprotection factor sunscreens in the uppression of actinic neoplasia. *Arch. Dermatol.* 1995; 131:170-175.
4. Yusuf N, Irby C, Katiyar SK, et al. Photoprotective effects of green tea polyphenols. *Photodermatol. Photoimmunol. Photomed.* 2007; 23: 48-56.
5. Koh HK. Preventive strategies and research for ultraviolet-associated cancer. *Environ. HealthPerspect.* 1995;103: 255-257.
6. Learn DB, Beasley DG, Giddens LD, et al. Minimum doses of ultraviolet radiation required to induce murine skin edema and immunosuppression are different and depend on the ultraviolet emission spectrum of the source. *Photochem. Photobiol.*1995; 62: 1066-1075.
7. De Gruijl FR, Forbes PD. UV-induced skin cancer in a hairless mouse model. *BioEssays* 1995; 17: 651-660.
8. Forbes PD. Relevance of animal models of photocarcinogenesis to humans. *Photochem.Photobiol.* 1996; 63: 357-362.
9. Slaga TJ, Budunova IV, Gimenez-Conti IB, Aldaz CM. The mouse skin carcinogenesis model. *J Investig Dermatol Symp Proc* 1996;1:151-6.
10. Berton TR, Fischer SM, Conti CJ, Locniskar MF. Comparison of ultraviolet lightinduced skin carcinogenesis and ornithine decarboxylase activity in sencar and hairless SKH-1 mice fed a constant level of dietary lipid varying in corn and coconut oil. *Nutr Cancer* 1996;26:353-63.
11. Davies RE, Forbes PD. Effect of UV radiation on survival of non-haired mice. *Photochem Photobiol* 1986;43:267-74.
12. Thomas-Ahner JM, Wulff BC, Tober KL, Kusewitt DF, Riggenbach JA, Oberyszyn TM. Gender differences in UVB-induced skin carcinogenesis, inflammation, and DNA damage. *Cancer Res* 2007;67:3468-74.
13. Slaga TJ, et al. Studies on the mechanisms involved in multistage carcinogenesis in mouse skin. *J. Cell. Biochem*. 1982;18:99-119.
14. Hara-Chikuma M, Verkman AS. Prevention of skin tumorigenesis and impairment of epidermal cell proliferation by targeted aquaporin-3 gene disruption. *Molecular and Cellular Biology* 2008;28(1): 326-332.
15. Ciurlea S, Dehelean CA, Ionescu D, Berko S, Csanyi Erszebet, Hadaruga N, Srinivas Ganta, Mansoor M. Amiji. A comparative study regarding melanoma activity of Betulinic acid on topical ointment vs. Systemic nanoemulsion delivery systems. *Journal of Agroalimentary Processes and Technologies*, 2010, 16(4), 420-426.
16. Kundoor V, Zhang, X, Bommareddy A, et al. Chemopreventive effects of sarcotriol on ultraviolet B-induced skin tumor development in SKH-1 hairless mice. *Mar. Drugs* 2007; 5:197-207.
17. Dwivedi C, Valluri HB, Guan X, Agarwal R. Chemopreventive effects of santalol on ultraviolet B radiation-induced skin tumor development in SKH-1 hairless mice. *Carcinogenesis* 2006; 27:1917-1922.
18. Willis I, Menter JM, Whyte HJ. The rapid induction of cancers in the hairless mouse utilizing the principle of photoaugmentation. *J. Invest. Dematol.* 1981; 76: 404-408.
19. Bowden GT. Prevention of non-melanoma skin cancer by targeting ultraviolet-B-light signaling.*Nat. Rev. Cancer* 2004; 4:23-35.
20. de Gruijl FR, Forbes PD. UV-induced skin cancer in a hairless mouse model. *Bioessays* 1995;17:651–60.
21. Murakami A, et al. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res*. 2000; 60: 5059-5066.
22. Seo HJ, et al. Inhibitory effects of the standardized extract (DA-9601) of Artemisia asiatica Nakai on phorbol ester-induced ornithine decarboxylase activity, papilloma formation, cyclooxygenase-2 expression, inducible nitric oxide synthase expression and nuclear transcription factor kappa B activation in mouse skin. *Int. J. Cancer* 2002; 100: 456-462.
23. Franco L, Doria D, Bertazzoni E, et al. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in pancreatic cancer. *Prostaglandins and Other Lipid Mediat* 2004; 73: 51-58.
24. Li W, Xu R, Jiang L, et al. Expression of cyclooxygenase-2 and inducible nitric oxide synthase correlates with tumor angiogenesis in endometrial carcinoma. *Med. Oncol*. 2005;22: 63-70.
25. Mordan LJ, Burnett TS, Zhang LX, et al. Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H10T1/2 fibroblasts. *Carcinogenesis* 1993; 14: 1555-1559.
26. Chin K, Kurashima Y, Ogura T, et al. Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and hepatocellular carcinoma cells. *Oncogene* 1997; 15: 437-442.

EVALUAREA MODIFICĂRILOR PARAMETRILOR TEGUMENTARI IN TUMORILE INIȚIATE CHIMIC ŞI FOTOCHIMIC LA LINIA MURINĂ SKH1

REZUMAT

Radiaţia medie sau ultravioletă B (UVB) cu o lungime de undă cuprinsă între 315-280 nm şi o energie per foton între 3.94-4.43 eV reglează răspunsul genetic la ultraviolete incluzând matricea metaloproteinazelor. Mai mult decât atât, acestea provoacă leziuni ale ţesutului conjunctiv iar pielea devine ridată şi îmbătrânită. Scopul acestui studiu a fost de a investiga evoluţia papiloamelor pe pielea şoarecelui la care s-a realizat iniţierea cu 7,12-dimethylbenzanthracene (DMBA) şi promovarea cu 12-O-decanoylphorbol-13-acetat (TPA), papiloame care au progresat mai rapid spre carcinom la şoarecii expuşi la UVB. Efectele biologice ale acestor doua tipuri de tumori (iniţiate chimic şi fotochimic) au fost evaluate timp de douăsprezece săptămâni pe şoareci SKH1 fără piele prin utilizarea unor tehnici non-invazive (tewameter, pH-metru tegumentar, măsurători privind melanina şi eritemul). S-a constatat că iradierea cu UVB afectează foarte mult parametrii care monitorizează şi caracterizează starea/condiţia pielii.

Cuvinte cheie: tumora, DMBA, TPA, UVB, şoareci SKH1, tehnică non-invazivă

ON THE ENTERIC NERVOUS SYSTEM'S ROLE IN PHYSIOLOGICAL ACTIVITIES OF THE GASTROINTESTINAL TRACT

ALINA SISU[1], CODRUTA PETRESCU[1], CARMEN TATU[2], LOREDANA STANA[1], R.F.TATU[3], A. MOTOC[1]

[1]Department of Anatomy and Embryology, University of Medicine and Pharmacy „Victor Babes" Timisoara
[2]Department of Functional Sciences, University of Medicine and Pharmacy „Victor Babes" Timisoara
[3]Department of Orthopaedics, Traumatology, Urology and Imagistics, University of Medicine and Pharmacy „Victor Babes" Timisoara

ABSTRACT

Intestinal smooth muscle is served by two types of intrinsic motor neurons, enteric excitatory neurons and inhibitors enteric neurons. Chemical mediators of the enteric nervous system were initially considered to be just as neurotransmitters acetylcholine and serotonin. Subsequent studies have added to the list purines such as ATP (adenosine triphosphate), peptides and amino acids such as GABA and VIP. More recently, NO was considered a neurotransmitter in the enteric nervous system, as in the CNS. The study was performed on 12 foetuses. There were made microscopic samples using trichrome Masson and Nissl stains. Submucous plexus, located in the submucosa, between circular muscle layer and mucosa muscle, is best developed in the small intestine where it plays an important role in controlling secretion. Keywords: neurotransmitters, foetuses, chemical mediators.

INTRODUCTION

Enteric nervous system is unusual in keeping its functions after central connections are removed. A variety of neuronal messengers (classical transmitters, gaseous messengers, amino acids and neuropeptide transmitters) are able to mediate and modulate gastric functions. Gastric nerves are either intrinsic to the gastric wall (have cellular corpus in intramural ganglia and thus belong to the enteric nervous system) or go to the stomach from the outside (extrinsic nerves), originating in the medulla oblongata, sympathetic ganglia and sensory ganglia. The endocrine and paracrine cells lining the stomach's mucosae (gastrin-secreting andsomatostatin cells), exocrine cells (parietal, mucous), smooth muscle cells and stromal cells are coordinated by neuronal messengers.

MATERIAL AND METHOD

The study was performed on 12 foetuses. There were made microscopic samples using trichrome Masson and Nissl stains.

RESULTS AND DISSCUTIONS

Although the enteric nervous system can operate independently of the central nervous system, the latter has an important role in coordinating the various functions of the enteric nervous system. Enteric nervous system is well connected to central autonomous network of CNS through both, motor and sensory sympathetic and parasympathetic pathways systems (Table I).

Table I: ENS role in physiological activities

Motility	
Tonic inhibition	suppression of spontaneous miogenic contractions by VIP or nitroxidergic neurons
Segmental contraction	mixture of lumen'substances of SI; suppression of inhibitors neurons may be involved in specific intestinal areas
Spreading the motor complex	series of cyclical contractions, each 1-2 hours, whose frequency is determined by the slow electrical wave generated by interstitial cells of Cajal; Cleansing interprandial and is initiated by motilin, somatostatin, prokinetic agents and opioids (Soudah et al., 1991) (1)
Primary oesophageal peristaltic	sequence of esophageal contractions central mediated and coordinated by nervous stimulants and inhibitors of the mienteric plexus and started by swallowing (Goyal and Paterson, 1989, Yamato et al., 1992b) (2,3)
Peristaltic by local reflex	motor sequence consisting of proximal contraction and relaxation distal to an intestinal distension stimulus; peristaltic movement is achieved by anal continuous displacement of the stimulus, being activated by the primary intrinsic afferent neurons containing 5HT (serotonin) (Furness and Bornstein, 1995)(4)

Received May 15th 2011. Accepted July 20th 2011. Address for correspondence: Assistant Professor Alina Sisu, MD, Department of Anatomy and Embryology, University of Medicine and Pharmacy "V. Babes" Timisoara, phone: 0724361671, e-mail: alinasisu@gmail.com; alinasisu@umft.ro

Huge peristaltic contraction	contraction of high amplitude, duration, representing a sustained peristaltic reflex, induced by cholera toxin and found in patients with spastic bowel syndrome; the electrical correlation is represented by the migration of action potential (Sarna, 1991, Mathias et al., 1976) (5,6)
Retrograd peristaltic contraction	involved in retrograde propulsion as in vomiting; neural circuit is unknown.
Sphincterial function	sphincters have intrinsic tonus by miogenic origin; relaxation is produced by or VIP and nitroxidergic neurons, contraction is provided by cholinergic neurons (Yamato et al., 1992a) (7)
Secretion	
Gastric acid secretion	stimulated by vagal activation of parietal cells and cells containing histamine or gastrin; inhibited by vagal activation of cells containing somatostatin
Pancreatic enzyme secretion	stimulated by activation colecistokinin induced of vagal primary afferent neurons and vagus vagal reflexes (Li and Owyang, 1994) (8)
Intestinal secretion	peristaltic activated, propagated complex motor and giant peristaltic contractions
Microcirculation	efferent sympathetic neurons produce ATP-mediated vasoconstriction; submucous intrinsic secretory neurons and axon reflexes cause vasodilation (Lundgren et al., 1989, Vanner and Surprenant, 1991, Evans and Surprenant, 1992) (9,10,11)
Immune and inflammatory responses	splanhnic primary afferent neurons innervate mucosal mast cells that degranulating and releasing inflammatory mediators and (Krumins et al., 1992, Stead, 1992, Shanahan, 1994) (12,13,14)

Sympathetic fibers arrived in the gut are adrenergic – postganglia fibers having the cellular corpus prevertebral ganglia. They have at least four targets in the gut:

- secretomotori neurons containing VIP;
- cholinergic presynaptic nervous endings;
- submucous blood vessels;
- gastrointestinal sphincters.

Cross section of the gastric body wall shows a magnification factor of 200 parietal layers from outside to inside (left> right) serous (with longitudinal vessels with erythrocytes in lumen caliber, on the cross section), muscle layers (longitudinal and circular, separated by a connective space and occupied by neuronal cell groups), submucosa, muscle and mucosae muscle. In submucosa are seen in dense groups of 3-10 nervous cells, evenly spaced on the circumference corresponding to Meissner - Henle submucos ganglia (black arrows). There are also seen rarified group of nervous cells but better represented numerically (blue border). Muscle motor neurons are located fully in the mienteric plexus, and most mucous neurons – in the submucous plexus (Furness and Costa, 1987) (15). Normal motility and secretory functions are dependent on the anatomical, chemical and functional integrity of this network that forms the enteric nervous system plexuses (Figure 1).

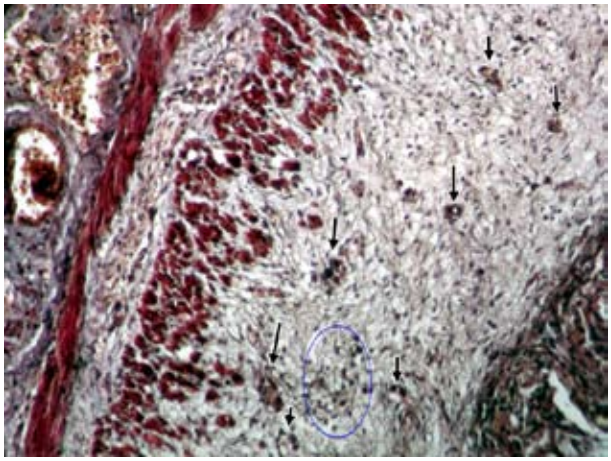

Fig. 1. Cross section through stomach; 20 cm human foetus. Trichrome Masson stain , x200. well-configured submucous ganglia (21 weeks)

Gastric wall layers contain, in humans, a rich cholinergic innervation: in the mucosa acetylcholinesterase positive fibers, around gastric glands and between the muscle fibers; in the submucosa are interconnected fibers and dispersed ganglionic cells, with strong positive reaction. The muscular layer contains the highest number of cholinergic fibers, grouped in bundles, and Auerbach plexus has the most intense positive reaction at the cellular level. Subserous layer contains a plexus having a positive reaction, and the external layer of vessels is rich in cholinergic fibers (Onicescu et al., 1975) (16) (Figure 2).

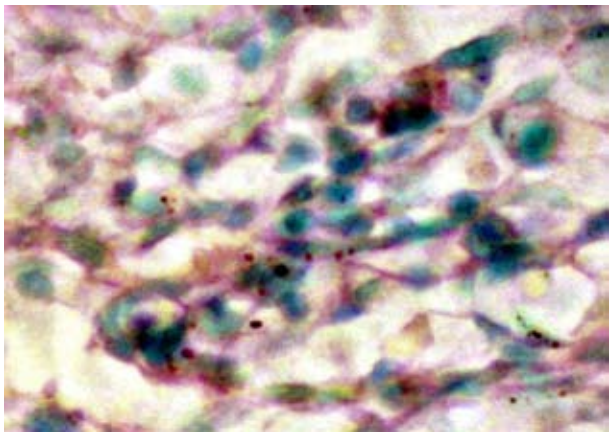

Fig.2. Oblique section through stomach; 18 cm human foetus; Nissl stain, x630. morphologic polimorphism on the gastric submucous plexus (19 weeks)

Details of submucous glands on 5 months foetus show submucous dense units (A) composed of variable individual cell-sized, which correlated directly with the amount of individual cytoplasm. These cells are emitting extra fine, short extensions, and long extensions that make up the periphery of a polygonal plexus. Internal area of the submucous layer (B) shows a deficit of morphological grouping on nerve cells that appear dispersed and with variable size, generally small (primitive neurons), confounding by fibroblasts and collagen fibers of submucoasae. Rudimentary plexuses are most obvious near the nervous cells (Figure 3).

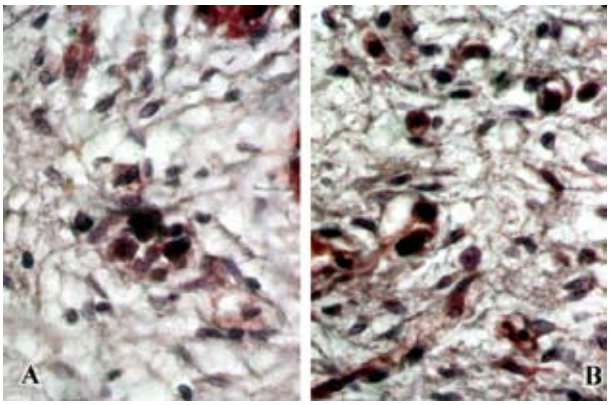

Fig. 3. Cross section through stomach; 20 cm human foetus; Trichrome Masson stain, x1000. submucous ganglia:
A: external layer; B: internal layer.

CONCLUSIONS

1. Vagal primary afferent neurons distributed in smooth muscle layer are sensitive to mechanical distension of the bowel, and lead information about certain motor intestinal physiological activities (Sengupta and Gebhart, 1994) (17).

2. Some vagal primary afferent neurons are sensitive to concentrations of glucose lights, amino acids or long chain fatty acids (Mei, 1985) (18), and others respond to a wide variety of mechanical and chemical stimuli.

3. Enteric glial cells produce interleukins and express MHC antigens / class II in response to cytokine stimulation (Ruhl and Collins, 1995) (19). They suggest an important role of enteric glial cells in modulating the inflammatory response to the gut.

4. Postnatal the central nervous system neuronal cell number will increase (rat, Altman and Das, 1966) (20) but the number of neurons decreases in visceral nervous system (Gabella, 1971) (21).

5. Although up to eight morphological types of neurons were identified in the enteric nervous system, there are two main types (Furness and Costa, 1987) (22,23,24,25):

• Type I neurons, having many processes in the form of club and one long thin process;

• Type II neurons, are multipolar and have many long and smooth processes.

6. Also it is considered that Cajal's interstitial cells acting like pacemakers, imposing the rate of intestinal contractions by affecting the electrical slow waves (Rich et al., 2002) (26); this assumption is supported by;

• ICC localization in smooth muscle layers where slow waves are generated;

• spontaneous activity of pacemaker type that has isolated ICC in the colon;

• disappearance of interruptions or slow electrical waves when ICC are removed or decoupled from intestinal smooth muscle (27,28,29).

REFERENCES

1. Soudah HC, Hasler WL, Owyang C. Effect of octreotide on intestinal motility and bacterial overgrowth in scleroderma. *N Engl J Med* 1991; 325: 1461-1467.
2. Goyal RK, Paterson WG. Esophageal motility. In: Schultz SG, ed. Handbook of physiology: a critical, comprehensive presentation of physiological knowledge and concepts. Sect. 6. The gastrointestinal system. Vol. 1. Motility and circulation. Bethesda, Md.: American Physiological Society 1989: 865-908.
3. Yamato S, Saha JK, Goyal RK. Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci* 1992; 50:1263-1272.
4. Furness J B, Bornstein J C, Murphy R, Pompolo S. Roles of peptides in transmission in the enteric nervous system. *Trends Neurosci* 1992; 15: 66-71.
5. Sarna SK. Physiology and pathophysiology of colonic motor activity. *Dig Dis Sci* 1991; 36:998-1018.
6. Mathias JR, Carlson GM, DiMarino AJ, Bertiger G, Morton HE, Cohen S. Intestinal myoelectric activity in response to live Vibrio cholerae and cholera enterotoxin. *J Clin Invest* 1976; 58:91-96.
7. Yamato S, Spechler SJ, Goyal RK. Role of nitric oxide in esophageal peristalsis in the opossum. *Gastroenterology* 1992; 103:197-204.
8. Li Y, Owyang C. Endogenous cholecystokinin stimulates pancreatic enzyme secretion via vagal afferent pathway in rats. *Gastroenterology* 1994; 107:525-531.
9. Lundgren O, Svanvik J, Jivegard L. Enteric nervous system. I. Physiology and pathophysiology of the intestinal tract. *Dig Dis Sci* 1989; 34: 264-283.
10. Vanner S, Surprenant A. Cholinergic and noncholinergic submucosal neurons dilate arterioles in guinea pig colon. *Am J Physiol* 1991; 261:G136-G144.
11. Evans RJ, Surprenant A. Vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation is mediated by the release of ATP. *Br J Pharmacol* 1992; 106:242-249.
12. Krumins SA, Broomfield CA. Evidence of NK1 and NK2 tachykinin receptors and their involvement in histamine release in a murine mast cell line. *Neuropeptides* 1992; 21:65-72.
13. Stead RH. Nerve remodelling during intestinal inflammation. *Ann N Y Acad Sci* 1992; 664:443-455.
14. Shanahan F. The intestinal immune system. În: Johnson LR, ed. Physiology of the gastrointestinal tract. 3rd ed. New York: Raven Press, 1994:643-84.

15. Furness JB, Costa M. The enteric nervous system. New York: Churchill Livingstone, 1987.
16. Onicescu D, Cuida I, Popescu M. Cholinergic innervation of the human stomach – Morphol Embryol (Bucur). 1975;21(2):93-7.
17. Sengupta JN, Gebhart GF. Gastrointestinal afferent fibers and sensation. In: Johnson LR, ed. Physiology of the gastrointestinal tract. 3rd ed. New York: Raven Press, 1994: 483-520.
18. Mei N. Intestinal chemosensitivity. *Physiol Rev* 1985; 65:211-237.
19. Rühl A, Collins SM. Enteroglial cells (EGC) are an integral part of the neuroimmune axis in the enteric nervous system (ENS). *Gastroenterology* 1995;108: Suppl:A680-A680.
20. Altman J, Das GD. Autoradiographic histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incoporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. *J Comp Neurol* 1996; 126: 337-390.
21. Gabella G. Neuron size and number in the myenteric plexus of the newborn and adult rat. *J Anat* 1971; 109: 81-95
22. Furness JB. Types of neurons in the enteric nervous system. *J. Autonom Nervous System* 2000; 81, 87- 96.
23. Furness JB, Costa M. Types of nerves in the enteric nervous system. *Neurosci* 1980; 5: 1-20.
24. Furness JB, Bornstein JC. The enteric nervous system and its extrinsic connections. In: Yamada T, ed. Textbook of gastroenterology. 2nd ed. Vol. 1. Philadelphia: J.B. Lippincott, 1995:2-24.
25. Furness JB, Kunze WA, Clere N. Nutrient tasting and signaling mechanisms in the gut. II. The intestine as a sensory organ: neural, endocrine and immuno responses. *Am J Physiol* 1999;277:G922-8.
26. Rich A, Hanani M, Ermilov LG, Malaysz J, Belzer V, Szurszewski JH, Farrugia G. Physiological study of interstitial cells of Cajal identified by vital staining. *Neurogastroenterology and Motility* 2002; 14:2-189.
27. Gabella G. Structure of muscles and nerves in the gastrointestinal tract. In: Johnson LR, ed. Physiology of the gastrointestinal tract. 3rd ed. New York: Raven Press, 1994:751-94.
28. Gabella G. Fine structure of the myenteric plexus in the guinea-pig ileum. *J Anat* 1972; 111: 69-97.
29. Costa M, Brookes SJ. The enteric nervous system. *Am J Gastroenterol* 1994; 89:Suppl:S129-S137.

EFECTELE HIPOXIEI ASUPRA PROLIFERARII SI APOPTOZEI CULTURILOR HUVEC IN VITRO

REZUMAT

Muşchiul neted intestinal este deservit de două tipuri de neuroni motori intrinseci, neuroni enterici excitatori şi neuroni enterici inhibitori, care în general proiectează oral şi anal, respectiv. Mediatorii chimici ai sistemului nervos enteric au fost consideraţi iniţial a fi doar neurotransmiţători precum acetilcolina şi serotonina. Studiile ulterioare au adăugat purinele pe listă, precum ATP, aminoacizii precum GABA şi peptidele precum VIP. Mai recent, NO a fost considerat drept neurotransmiţător în sistemul nervos enteric, precum în SNC. Studiul a fost realizat pe 12 feti. Au fost realizate preparate microscopice utilizand tehnicile de colorare tricrom Masson si Nissl. Plexul submucos, localizat în submucoasă, între stratul muscular circular şi musculara mucoasei este cel mai bine dezvoltat în intestinul subţire unde joacă un rol important în controlul secreţiei.

Cuvinte cheie: neurotransmitatori, feti, mediatori chimici.

AMBULATORY SYSTOLIC AUGMENTATION INDEX-A NEW ARTERIAL RIGIDITY MARKER FOR CARDIOVASCULAR RISK STRATIFICATION

IONESCU V.A, GEORGIANA IONESCU, DANA BÂIBÂȚĂ, SILVIA MANCAŞ, ŞT. I. DRĂGULESCU

University of Medicine and Pharmacy "Victor Babeş" Timişoara

ABSTRACT

Ambulatory augmentation systolic index (AASI) derived from the 24h blood pressure monitoring is one of the new markers of arterial stiffness. Our study aims to analyze possible associations between AASI and global cardiovascular risk. Means and method: we included 130 asymptomatic subjects that were evaluated through several perspectives: global cardiovascular risk - SCORE risk charts; cardio-metabolic risk: traditional cardiovascular risk factors, metabolic syndrome defined by the IDF criteria; hemodynamic profile: 24h blood pressure monitoring with the derived data - pulsed pressure (PP); arterial wall profile: the carotid-femoral artery pulsed wave velocity (C-F PWV by the Complior method) and AASI, derived from 24h blood pressure monitoring. Results: we found statistically signifficant correlations between AASI and 24h-PP: $r = 0.46$, $p < 0.001$; we also obtained signifficant differences of mean AASI in subjects with and without metabolic syndrome, $t(128) = -2.63$, $p<0.01$. Mean AASI variations according to the number of cardiometabolic risk factors were not signifficant. We found a strong corelation between C-F PWV and SCORE: $r=0.404$, $p < 0.001$, but not between AASI and SCORE. There also was no significant correlation between AASI and C-F PWV. Conclusions: we appreciated the vascular remodelling of asymptomatic patients at risk by using non invasive methods of evaluating arterial stiffness: C-F PWV and AASI. AASI behaviour related to the hemodynamic variable of the cardivascular risk, but not to the cardiometabolic profile. As there are no corelations found between C-F PWV and AASI, the two markers can not substitute one another; AASI identifies the hemodynamic risk, whereas PWV gives us extra data regarding the contribuiton of metabolic syndrome on the vascular remodelling. In light of this data, it becomes clear that SCORE charts are no longer sufficient for risk stratification, and other parameters should also be taken into account.

Key words: risk stratification, asymptomatics at risk, arterial stiffness, ambulatory augmentation systolic index, carotid-femoral pulsed wave velocity, pulsed pressure.

THEORETICAL ASPECTS

Over the past 5-6 years, the systolic augmentation index derived from 24 h monitoring of blood pressure begun to be interpreted as a new marker of arterial rigidity. The relationship between the index and arterial stiffness is based on following observations:

First of all, Li Y et al (1), after analyzing 166 normotensive Asiatic subjects, proved a direct and statistically significant relationship between systolic augmentation index and carotid-femoral pulsed pressure.

Second of all, it seems that this index is somewhat related to the degree of nocturnal reduction in blood pressure. Schillaci et al (2) has proved that the systolic augmentation index in hypertensive patients included in the non-dipper category was significantly higher than in patients with dipper hypertensive profile: 0.44 ± 0.20 vs. 0.29 ± 0.15; $p<0.001$.

The significant association between the systolic augmentation index and arterial rigidity might be explained by the connection with the same variables: age, arterial distension pressure, heart rate and nocturnal reduction in blood pressure. If this is correct, could the degree of nocturnal drop in blood pressure be regarded as a marker of arterial rigidity? In Schillaci's study (2), the non-dipper hypertensive patients have had higher velocities of the carotid-femoral pulsed wave than the dippers, but the difference was not statistically significant after age adjusting: 9.5 ± 2 m/s vs. 9.2 ± 2 m/s, $p=0.30$.

Third of all, the variance of arterial rigidity depending on the arterial distension pressure is a non-linear curve. This means that, both arterial rigidity and pulsed wave velocity comport great variations throughout the cardiac cycle. Therefore, the use of the systolic/diastolic regression slope in expressing the ambulatory systolic augmentation index becomes an independent parameter: non-dependent on the pressure. In order to sustain this theory, Gavish B et al (3) have proved that this systole-diastolic slope quantitatively expresses the relative increase of arterial stiffness throughout the systole and it is independent of the pressure within the vessel.

Is it possible then, that the two markers of arterial rigidity express different aspects? It seems so: the pulsed wave velocity estimates the arterial rigidity at one point on the pressure-volume curve, whereas the ambulatory systolic augmentation index estimates intrinsic arterial rigidity.

Given the scientific context, we have designed this transversal study regarding the practical use of the systolic augmentation index for the characterization of the arterial function in high risk asymptomatic patients.

Received August 16th 2011. Accepted September 15th 2011. Address for correspondence: Ionescu Vlad Alexandru, MD, "Victor Babes" University of Medicine and Pharmacy Timisoara, Eftimie Murgu Square No. 2A, 300041, phone/fax: +40256220479, phone: +4 0722221802, e-mail: i_andru@yahoo.com

Hypothesis of the study: In order to be able to evaluate the implication of a new parameter in cardiovascular risk stratification of asymptomatic patients, we issued the hypothesis that ambulatory systolic augmentation index might reflect arterial stiffness, a condition associated with cardiovascular events.

The main objective of the study was to analyze possible associations between ambulatory systolic augmentation index and global cardiovascular risk, with serious implication in the primary prophylaxis of cardiovascular disease.

MEANS AND METHOD

We included in our study high risk asymptomatic subjects, assessed according to the cardiovascular risk factors. The cases were stocked in the archive of the Preventive Cardiology and Cardiovascular Rehabilitation Clinic of the Cardiovascular Disease Institute, Timisoara. The including and excluding criteria are presented in Table I. All patients signed the informed consent and the study was approved by The Ethic's Committee at the Cardiovascular Disease Institute, Timisoara.

Table I: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
• Asymptomatic subject acc. to SEC	• Signifficant valvular heart disease
• Synus rythm	• Arrhythmias
• Preserved ejection fraction	• Perripheral obstructive arterial disease
• Normotensive/ dipper hypertensive profile	• Congenital heart disease

Data collection: we used the clinical charts for data collection; the analyzed parameters included demographic data, anthropometric data, anamnesis, complete clinical examination and paraclinical evaluation.

The evaluation protocol was designed in order to define the global cardiovascular risk of the asymptomatic subject and to characterize their arterial function.

A. The risk for fatal cardiovascular event within the next 10 years: we used the SCORE chart- the high risk European population chart. The quantified data were: age, sex, systolic blood pressure (mmHg), smoking, total cholesterol (mg/dl). According to the SCORE risk, the asymptomatic subjects were classified as: SCORE<5% and SCORE>5%.

B. Cardiometabolic risk: for the cardiometabolic risk stratification we included traditional cardiovascular risk factor assessment, as well as the basal characteristics of the group: age, sex, waist circumference, body mass index (kg/m^2).

Arterial hypertension was defined according to The 2007 Guidelines for Management of Arterial Hypertension (4): SBP≥ 140 mmHg and/or DBP≥ 90 mmHg or antihypertensive treatment; this corresponds to a 24 h mean arterial blood pressure > 120/80 mmHg.

Smoking status: number of cigarettes smoked daily. Non-smoking: at least 4 weeks from smoking cessation.

Type II diabetes mellitus was defined according to a fasting plasma glucose ≥ 126 mg/dl or andt-diabetic therapy.

Hypercholesterolemia, according to the National Cholesterol Education Program-Adult Treatment Panel III 2001 (5) as defined for total cholesterol values higher than 200mg/dl. Because target level of total cholesterol in primary prophylaxis is below 190mg/dl, all values above this were considered a major risk factor. Furthermore, hypolipemiant therapy was also considered diagnostic criteria for hypercholesterolemia.

The metabolic profile: pro-atherogenic dyslipidemia was defined by NCEP-ATP III (5). We took into account the recommendations of The Guidelines on Cardiovascular Disease Prevention (6) in order to characterize, in the view of primary prophylaxis, the "healthy" condition: LDLc<115mg/dl, HDLc>40mg/dl, TG<150mg/dl. Metabolic syndrome was defined according to the IDF 2005 criteria (Table II).

Table II: Metabolic syndrome: definition criteria

CRITERIA	NCEP-ATP III	IDF
Abdominal circumference(cm)	>88 (F),>102 (M)	>80 (F),>94 (M)*
Arterial blood pressure (mmHg)	≥ 130/80	≥ 130/80
Fasting plasma glucose (mg/dl)	> 110	> 100
Triglyceride (mg/dl)	>150	>150
HDL-c (mg/dl)	<50(F),<40(M)	<50(F),<40(M)

* mandatory criteria for IDF classiffication
Note: if IMC ≥ 30 kg/m^2, waist measurement is no longer necessary according to the IDF

C. Hemodynamic risk: measurement of arterial blood pressure was done at the brachial artery site, bilateral, in respect to the recommendations of The European Society of Hypertension Guideline (7): sitting position, after 5 minutes of rest. We used the media from 3 consecutive measurements.

Ambulatory monitoring of the arterial blood pressure: the data regarding the 24h blood pressure profile was collected using the BTL-08 ABPM program automatic readings of the blood pressure were performed every 15 minutes during the day (7am-11pm) and every 30 minutes during the night (11pm-7am). The monitoring was repeated if it experienced more than 30% artefacts. Throughout the entire monitoring period, all medication was withdrawn. From the ambulatory blood pressure monitoring protocol, we used the following parameters: 24h SBP, 24h PP, 24h MABP. We considered as cut off value for nocturnal reduction of blood pressure 10%. Those with more than 10% nocturnal reduction in blood pressure were considered as dippers, thus becoming possible candidates for our study.

D. Arterial function was appreciated through the following measurements:

- Carotid-femoral pulsed wave velocity: C-F PWV
-Ambulatory systolic augmentation index: AASI

For determining the C-F PWV we used the Complior SP System. PWV represents the pulsed wave generated by each ventricular ejection; it is then propagated along the arterial tree, according to the Moens-Korteweg equation, $PWV^2 = (E \times h)/(2r \times rho$, where E=the elasticity module of the arterial wall, h=arterial

stiffness, r=blood density, rho=the internal diameter of the femoral artery. In normal physiologic conditions, the h/r ratio is constant; therefore, PWV is directly related with the elasticity module of the arterial wall.

C-F PWV measurement was done by simultaneous registration of the 2 wave forms: carotid and femoral wave forms. C-F PWV was calculated as the ratio between the distance traveled between 2 selected points and the time difference between the 2 wave forms: c-f PWV(m/sec) = d/ Δ t.

Note: the C-F PWV measurement was obtained after at least 24 hours cessation of alcohol and coffee intake; the last pharmacological treatment was administrated 12h prior to the exam.

The AASI was calculated using the linear regression, as a ponderate function of successive evaluation of arterial blood pressure within a 24 hours interval (1). For each subject, we analyzed the regression slope of the diastolic blood pressure in relation to the systolic blood pressure. AASI was calculated after the formula (8):

AASI = 1 – regresion slope of DBP/SBP

The more rigid the arterial wall, the more tendency of the regression slope towards 0, and of AASI towards 1 (9).

STATISTICAL ANALYSIS

The statistical analysis performed used the EPIINFO program, 6.0, 2001 version; data was analyzed using the SPSS program, the 2010 18th version; it was electronically stocked using the 97-03 version of The Microsoft Excel program and the PASW program, 18th version 2010. The cut off value for the statistical significance was $p<0.05$; a very strong statistical significancy was considered for a $p<0.01$.

RESULTS

We included 214 consecutive patients evaluated in the Preventive Cardiology and Cardiac Rehabilitation Clinic of the Cardiovascular Disease Institute Timisoara between May 2009-May 2011: 109 females, 105 males, and 1.03 to 1 ratio. From this 214 group of patients, 130 were asymptomatic subjects (mean age 58.62±8.39 years old, 36.15 males) at cardiovascular risk, according to the criteria mentioned above, who signed the approval for the study. 66.15% of them had a high SCORE ≥ 5%. The prevalence of traditional cardiovascular risk factors was:

- Essential hypertension 88.5 % (n=115 subjects)
- Type II diabetes mellitus 23.1 % (n= 30 subjects)
- Hypercholesterolemia 60.0 % (n= 78 subjects)
- Smokers 46.2 % (n= 60 subjects)
- Family history of premature CV disease 56.2 % (n= 78 subjects)

The metabolic profile of the group consisted of these mean (mg/dl):

- Total cholesterol 223.86 ± 55.16 mg/dl
- Triglycerides 184.62 ± 94.61 mg/dl
- LDLc 139.73 ± 50.59 mg/dl
- HDLc 46.48 ± 13.39 mg/dl
- Fasting plasma glucose 107.42 ± 25.34 mg/dl

Metabolic syndrome defined according to the IDF criteria was recorded in 52.3% of the subjects. According to the protocol, the entire lot had 24h blood pressure monitoring. Mean SBP was 151.48± 12.16 mmHg. Mean PP was 63.85 ± 10.72 mmHg.

The relationship between AASI and the hemodynamic profile: mean AASI was 0.58 ± 0.12, with a maximum of 0.92 and a minimum of 0.36. Individual value had a Gaussian-like distribution. We found statistically significant correlations between AASI and 24h-PP: $r = 0.46$, $p < 0.001$; the association size between the 2 variables was 21.9% (Figure1).

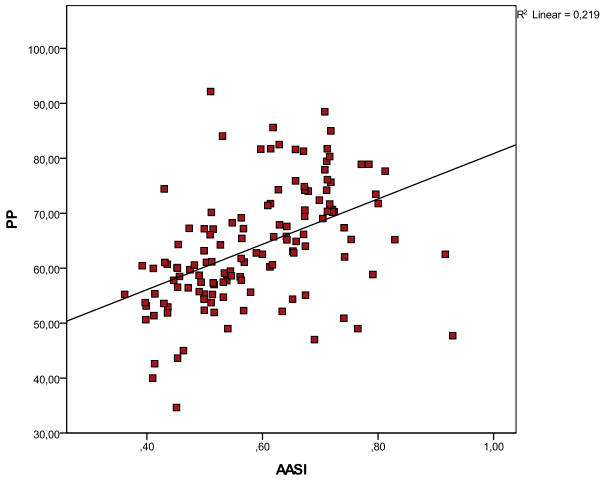

Fig.1. Correlation graphic between AASI and PP

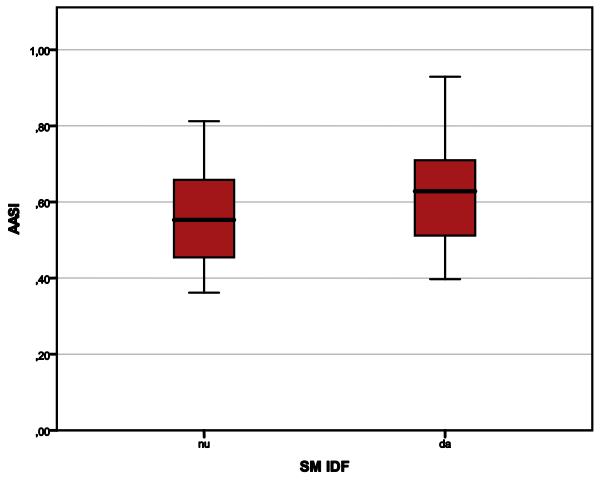

Fig.2. AASI and metabolic syndrome

The relation between the cardiometabolic risk score and AASI

a. The relation between AASI and metabolic syndrome: we obtained significant differences of the mean AASI in subjects with and without metabolic syndrome, $t(128)=-2.63$, $p<0.01$ (Fig. 2).

b. The analysis of AASI behavior and the cardiometabolic

risk factors: SCORE≥ 5%, LDLc ≥ 115 mg/dl, TG ≥ 150 mg/dl, type II diabetes mellitus. The highest frequency of the cases had 2 (33.8%), respectively 3 of these criteria (30.8%). Mean AASI ranged between 0.45 and 0.59 (Table III). We applied the ANOVA test to see if there are any statistically significant differences of the mean AASI according to the number of present criteria; we found none, p>0.05 (Table IV).

Study of the AASI variation according to the global high cardiovascular risk: SCORE> 5%, LDL > 115 mg/dl, TG > 150 mg/dl and type II diabetes mellitus proved no statistical significant difference (p>0.05). The linear regression model used to prove that that these 4 variables are predictors of the AASI evolution failed (Table V).

Table III. Mean AASI according to the number of cardiometabolic risk factors

No. of criteria	No	Mean	Standard deviation	Minimum	Maximum
0	4	.45	.08 2	.36	.56
1	21	.60	.12	.41	.78
2	44	.59	.12	.39	.91
3	40	.59	.12	.39	.92
4	21	.57	.10	.43	.80
Total	130	.58	.12	.36	.92

Table IV. Comparing mean AASI according to the number of cardiometabolic risk factors

Criteria	No	Mean	Standard deviation	P
AASI >= 2	105	.58	.12	>0.05
< 2	25	.58	.12	

Criteria	No	Mean	Standard deviation	P
AASI >= 3	61	.58	.11	>0.05
< 3	69	.59	.12	

Table V. Liniar regression model for AASI

SCORE risk and the behavior of arterial stiffness markers: PWN and AASI. Mean C-F PWV was 10.63± 2.12 m/sec. We found a strong significant correlation between c-f PWV and SCORE: r=0404, p < 0.001 (Figure 3).

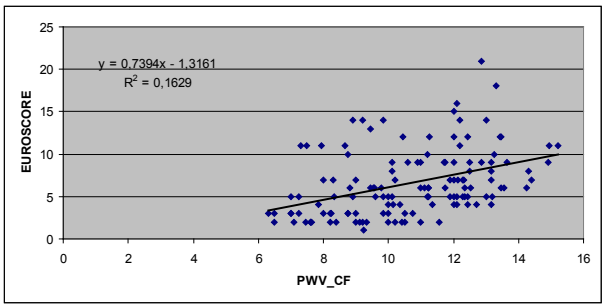

Fig. 3. C-F PWV and SCORE risk: corelation graphic

Mean AASI was 0.58 ± 0.12. The association between AASI and the SCORE risk was not statistically significant: 0.59 ± 0.14 in subjects with SCORE<5% and 0.58 ± 0.11 in subjects with SCORE≥ 5%, p>0.05 (Figure 4).

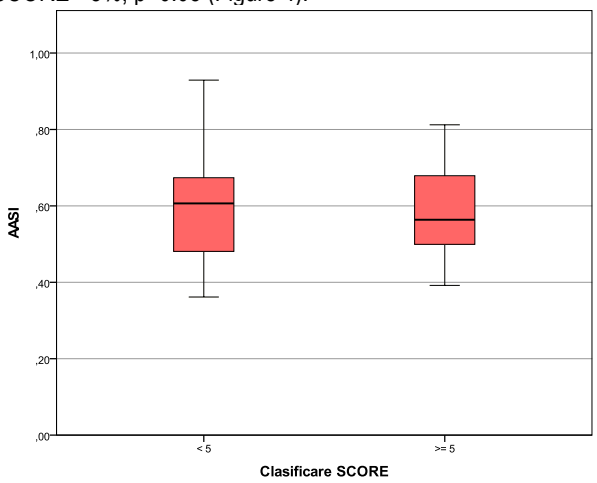

Fig.4. Relation between AASI and SCORE risk

The correlation between the 2 markers of arterial rigidity AASI and c-f PWV was not significant, p>0.05.

DISCUSSION

In 1914, Mac William and Melvin proved that the reduction in arterial elasticity influenced DBP behavior and the relation with

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.141[a]	.020	-.011	.1224664	.020	.634	4	125	.639
2	.135[b]	.018	-.005	.1220759	-.002	.198	1	125	.657
3	.124[c]	.015	.000	.1217770	-.003	.379	1	126	.539
4	.099[d]	.010	.002	.1216463	-.006	.725	1	127	.396
5	.000[e]	.000	.000	.1217698	-.010	1.262	1	128	a. Predictors: (Constant), FR_DZ, TG150, riscSCORE5, LDL115

b. Predictors: (Constant), FR_DZ, TG150, EUROSCORE5

c. Predictors: (Constant), FR_DZ, TG150

d. Predictors: (Constant), FR_DZ

SBP (10). Considering this, it means that AASI refect the distinct relation between DBP and SBP, during the night.

However, the connection between AASI and the mechanical properties of the arterial wall remains controversial.

Our study clearly proved the association relation between AASI and 24h PP, among a heterogenous group of asymptomatic subjects with cardiovascular risk, including both normotensive as well as hypertensive subjects.

Therefore, from the 2 methods of evaluating arterial stiffness, which one is the most accurate in determining the risk and prognosis of these subjects? Dublin Outcome Study (8) shades light onto some of the aspects:

- AASI was a better predictor of cardiovascular mortality than PP in normotensive patients compared to hypertensive patients, but,

- PP was a prognostic factor among hypertensive patients.

Elizabeth S. Muxfeldt (11) has studied the arterial wall rigidity in a group of 391 hypertensive patients (resistant hypertension), using 3 methods: PP, AASI and PWV (the Complior method). PP pressure was best correlated with arterial wall stiffness in these patients.

Recent data talks about the correlation between C-F PWV, AASI and PP, thus justifying the evaluation of arterial function even among adolescents (12). The best evidence was obtained for 24h PP, which was correlated with: arterial stiffness measured by PWV, target organ damage (left ventricular mass) and the presence of hypertension.

It is important to underline that some aspects coming from 3 cohort prospective studies regarding the significant association between AASI and fatal/nonfatal cardiovascular events (8,13,14): the relation between AASI and cardiovascular mortality is of a U-curve type; furthermore, AASI was proved to have a predictive value for cerebrovascular event in normotensive patients.

Another important issue worth discussing is that this predictive value of AASI for cerebrovascular events should not be regarded only through the hemodynamic perspective, but also through the cardiometabolic one. Leoncini G et al (15), by analyzing the association between AASI and metabolic profile in 156 hypertensive non-diabetic patients, has reached the following conclusions:

- the prevalence of high AASI was significantly higher among patients with metabolic syndrome (p=0.02)

- the presence of metabolic syndrome doubles the risk for AASI augmentation.

Our study has found significantly higher values for AASI among the asymptomatics with metabolic syndrome defined by the IDF criteria, but not in asymptomatics with metabolic syndrome defined by the ATP III criteria. The mandatory criterion for the IDF is the waist circumference or a BMI≥30 kg/m^2. In our study, we obtained a positive and significant correlation between PWV, as a marker of arterial stiffness, and BMI. Our model though, designed in order to analyze the AASI variance from the cardiometabolic perspective, was not valid. This is why we could use AASI and 24 h PP in our algorhythm to evaluate arterial function in asymptomatic subjects with hemodynamic risk: associated cardiometabolic risk does not influence in any was AASI behavior.

We want to underline that in our study the statistical procedure has discarded age from the equation analysis of arterial wall rigidity. This because mean age was 58.62 years old, an age when there is a normal remodelling of the arterial wall.

The PROOF study (16) was centred on the influence of age and blood pressure on cardiovascular events related to increased arterial stiffness; it only enrolled subjects over 65 years of age. At this age, the conclusions were that AASI varied significantly according to traditional risk factors (hypertension, obesity, hypercholesterolemia and diabetes); there was a negative correlation between AASI and HDLc; there were no correlations found between AASI and C reactive protein. However, the multivariate analysis showed that only hypertension and diabetes mellitus contributed to AASI augmentation in subjects older than 65. The 2 years follow up showed that, even in normotensive patients, AASI augment with age. This process is not so obvious among hypertensive patients under optimal management of 24h blood pressure.

Study limitations: the design of our observational cross-sectional study gives us the results of an analysis at one point in time, as any such study. Obviously, longitudinal follow-up over several years of the interactions between different indices of arterial stiffness would bring more information in regards of what the arterial wall dysfunction really means.

CONCLUSIONS

1. We appreciated the vascular remodelling of asymptomatic patients at risk by use of 2 non invasive methods of evaluating arterial stiffness: c-f PWV and AASI.

2. AASI behavior related with the hemodynamic variable (24h PP) of the cardiovascular risk.

3. AASI variance was not influenced by the cardiometabolic risk profile.

4. As there are no correlations between c-f PWV and AASI, the two markers can not substitute one another.

5. AASI identifies the hemodynamic risk, whereas PWV gives us extra data regarding the contribution of metabolic syndrome at the vascular remodelling of the asymptomatic patient with traditional cardiovascular risk factors.

6. Cardiovascular risk stratification based only on the SCORE charts is insufficient

7. The use of biomarkers for arterial stiffness allows not only a more accurate characterization of the risk profile in asymptomatic subjects, but also a more specific approach of the risk management.

REFERENCES

1. Li Y, et al. Ambulatory arterial stiffness index derived from 24 hour ambulatory blood pressure monitoring. *Hypertension*, 2006; 47: 359-64.
2. Schilacci G, et al. Ambulatory arterial stiffness index is not a specific marker of reduced arterial compliance. *Hypertension*, 2007; 49: 986-91.

3. Gavish B, et al. What Arterial Property Is Expressed By the Systolic-Diastolic Pressures Relationship? A Model View: Pp.14.41. Journal of Hypertension. 28:e260, June 2010.
4. Mancia G, et al. The 2007 Guidelines for the Management of Arterial Hypertension. *Europ.Heart J*, 2007; 28: 1462-536.
5. Scott GM, et al. Implications of Recent Clinical Trials for the National Cholesterol Education Program Adult Treatment Panel Guidelines. *Circulation*, 2004; 110: 227-39.
6. Ian Grahan, et al. The Guidelines on Cardiovascular Disease Prevention. *European J. Cardiovascular Prevention and Rehabilitation*, 2007; 4 (2).
7. Mancia G, et al. Ghidul Societătii Europene de HTA pentru evaluarea si tratamentul HTA. *Rev. Română Cardiologie XVIII*, 2003; 3:1-42.
8. Dolan E et all- Ambulatory arterial stiffness index as a predictor of cardiovascular mortality in the Dublin Outcome Study. Hypertension 2006,47,3,365-70
9. Adiyaman A, et al. Determinants of the ambulatory arterial stiffness index in 7604 subjects from 6 populations. *Hypertension* 2008; 52(10): 38-44.
10. Mac William, et al. Systolic and diastolic blood pressure estimation with special reference to the auditory method. *BMJ*, 1994; 2:693.
11. Muxfeldt ES, et al. Ambulatory arterial stiffness or pulse pressure? *Hypertension Research* 2008; 31: 607-13.
12. Stergiou GS, et al. Ambulatory arterial stiffness index, pulse pressure and pulse wave velocty in children and adolescents. Hypertension Research, 2010; 33: 1272-77.
13. Hansen TW, et al. Ambulatory arterial stiffness index predicts stroke in a general population. *J. Hypertension,* 2006; 24: 2247-53.
14. Kikuya M, et al. Ambulatory arterial stiffness index and 24h ambulatory pulse pressure as predictors of mortality in Ohasama. Japan. *Stroke*, 2007; 38: 1161-66.
15. Leoncini G, et al. Metabolic syndrome and ambulatory arterial stiffness index in non-diabetic patients with primary hypertension. *Journal of Human Hypertension*, 2007; 21: 802-07.
16. Gosse P, et al. Components of arterial stiffness in a population of 65 year-old subjects: PROOF study. *J.Hypertens,* 2008; 26:1138-46.

INDICELE AMBULATORIU DE AUGMENTARE SISTOLICĂ - UN NOU MARKER DE RIGIDITATE ARTERIALĂ FOLOSIT PENTRU STRATIFICAREA RISCULUI CARDIOVASCULAR

REZUMAT

Indicele ambulatoriu de augmentare sistolica (AASI), derivat din monitorizarea Holter 24 de ore a tensiunii arteriale este un nou marker al rigiditatii arteriale. Studiul nostru are ca scop analiza unor posibile asocieri intre AASI si riscul cardiovascular total. Material si metoda: am inclus in studiu 130 de subiecti asimptomatici evaluati din mai multe perspective: risc cardiovascular global - riscul SCORE; riscul cardio-metabolic - factorii de risc cardiovascular traditionali, sindromul metabolic definit dupa criteriile IDF; profilul hemodinamic: monitorizare Holter 24 de ore a tensiunii arteriale si presiunii pulsate; profilul peretelui arterial: viteza undei pulsate carotida-femurala (PWV C-F, metoda Complior) si AASI, derivat din monitorizare Holter 24 de ore. Rezultate: am gasit corelatii semnificative statistic intre AASI si PP: r=0,46; p<0,001; de asemenea, diferente semnificative au fost demonstrate si intre AASI la subiectii cu sindrom metabolic vs AASI la cei fara sindrom metabolic, t(128)=-2.63, p<0.01. Variatiile mediei AASI in functie de numarul de factori de risc cardiometabolic nu au fost semnificative. Am dmonstrat o corelatie puternica intre PWV C-F si riscul SCORE: r=0.404, p < 0.001, dar nu si intre AASI si SCORE. De asemenea, nici asocierea AASI - PWV nu a fost semnificativa. Concluzii: folosind metode noninvazive - AASI, PWV C-F, am apreciat afectarea arteriala la asimptomaticii la risc. Comportamentul AASI s-a relationat cu componenta hemodinamica a riscului cardiovascular, dar nu si cu componenta cardio-metabolica. In plus, deoarece nu exista corelatii intre AASI si PWV, cei doi trebuie priviti ca doi markeri independenti, care nu se pot substitui unul cu celalalt; AASI defineste riscul hemodinamic, in timp ce PWV ne ofera informatii cu privire la impactul vascular al componentei metabolice a riscului. Astfel, devine evidenta concluzia ca riscul SCORE este deja insuficient pentru stratificarea fina a riscului cardiovascular, si ca este nevoie si de alte metode complementare pentru o mai buna intelegere a riscului global al asimptomaticilor.
Cuvinte cheie: stratificarea riscului, asimptomatic la risc, rigidiatate arteriala, indicele ambulatoriu de augmentare sistolică, velocitatea undei pulsate carotida-femurala, presiune pulsata.

THE RELATIONSHIP BETWEEN HYPERTENSIVE RETINOPATHY AND CORONARY ARTERY DISEASE

OVIDIU BORUGA, IOANA ZOLOG

Victor Babes University of Medicine and Pharmacy Timisoara, Ophthalmology Clinic Timisoara

ABSTRACT

Aim of study. To assess the relationship between hypertensive retinopathy and coronary artery disease in a population from Timis County, Romania.

Methods. We conducted an observational study that included 600 patients (382 men and 218 women), aged 40-65 years who were examined for retinal microvascular changes by retinal photographs within the Ophthalmology Clinic Timisoara between March 2008 and May 2011. We included in our study patients with hypertension on the basis of systolic and diastolic blood pressure measured in three consecutive visits (more than 140 systolic or/and 90 diastolic mmHg, measure in a rest condition with a mercury sphygmomanometer).

Results. After initial evaluation of blood pressure 360 patients (60%) had first stage hypertension, 180 patients (30%) had second stage hypertension and 60 patients (10%) had third stage hypertension. No patient had malignant hypertension at initial evaluation.

We found hypertensive retinopathy in 210 patients (35%): focal and general arteriolar narrowing in 108 patients (18%); arteriovenous nicking in 60 patients (10%); microaneurysms, haemorrhages and exudates (retinopathy) in 42 patients (7%). No patient had disc swelling or optic nerve oedema. The presence of any retinopathy sign predicted a three fold higher risk for coronary artery disease (RR 3.71; 95% CI 2.76 to 4.98), with higher risk for patients with retinopathy (RR 4.19; 95% CI 3.41 to 5.15) as compared to AV nicking (RR 2.25; 95% CI 1.69 to 2.97) and arteriolar narrowing (RR 1.23; 95% CI 0.88 to 1.71). The incidence of coronary artery disease was higher in patients with second and third stage hypertension, in patients with a poor control of blood pressure, despite treatment. Incidence of hypertensive retinopathy was higher in women, while the incidence of coronary artery disease was higher in men.

Conclusion. Our study showed that evaluation of hypertensive retinopathy is important in hypertensive patients because the presence of any modification (arteriolar narrowing, arteriovenous nicking and retinopathy) is associated with three fold higher risk of developing coronary artery disease. The incidence of hypertensive retinopathy is higher in women, but men had higher incidence of coronary artery disease. Patients with retinopathy (microaneurysms, hemorrhages and/or exudates) are at highest risk. It is therefore important to undergo adequate control of blood pressure and other risk factor.

Key words: hypertensive retinopathy, coronary artery diseases

INTRODUCTION

Keith, Wagener, and Barker classification has been used to assess retinal changes associated with hypertension since 1939 (1). The recent guidelines of Joint National Committee and European Society of Hypertension still recommend routine ophthalmoscopic examination to detect signs of retinopathy in people with hypertension for the purposes of risk stratification and treatment decisions, although its value is being questioned. The guidelines emphasize that hypertensive retinopathy, together with left ventricular hypertrophy and renal impairment may be considered an indicator of target organ damage, suggesting that physicians should consider a more aggressive approach in managing these patients (2, 3).

Hypertension has profound effects on the structure and function of the eye. First, the retinal, choroidal, and optic nerve circulations undergo a series of pathophysiological changes in response to raised blood pressure, resulting in a range of clinical signs referred to as hypertensive retinopathy, hypertensive choroidopathy, and hypertensive optic neuropathy. Second, hypertension is an important risk factor for the development of potentially blinding vascular diseases of the eye, including retinal vein and artery occlusion, retinal-arteriolar emboli, and diabetic retinopathy. Finally, hypertension might be a pathogenic factor for non-vascular ocular diseases, including two of the leading causes of blindness-glaucoma and age-related macular degeneration. Hypertensive retinopathy refers to retinal microvascular signs related to increased blood pressure (4, 5).

Hypertensive retinal vascular signs can be broadly classified into arteriolar changes (generalized arteriolar narrowing, focal arteriolar narrowing, arteriovenous nicking and arteriolar wall opacification) and more advanced retinopathy lesions (microaneurysms, blot and flame-shaped hemorrhages, cotton-wool spots, hard exudates and optic disk swelling). With the exception of disk swelling, these signs can be detected fairly frequently in adult populations, even in persons without a known history of hypertension (4).

Many studies have reported a strong association between various hypertensive retinopathy signs and both subclinical and clinical cerebrovascular disease (6-14) and stroke mortality (15).

We conducted an observational study to asses the relationship between hypertensive retinopathy and coronary artery disease in a population from Timis County, Romania.

Received 15th August. Accepted 15th September. Address for correspondence: Ovidiu Boruga, MD, Ophthalmology Department, University of Medicine and Pharmacy "Victor Babes" Timisoara, Eftimie Murgu Sq. No. 2A, 300041 Timisoara, RO, E-mail: ileanazolog@yahoo.com

METHODS

Study population

Our study included 600 patients (382 men and 218 women), aged 40-65 years who were examined for retinal microvascular changes by retinal photographs in Ophthalmology Clinic Timisoara between March 2008 and May 2011. We included in our study patients with hypertension on the basis of systolic and diastolic blood pressure measured at three consecutive visits (more than 140 systolic or/and 90 diastolic mmHg, measure in a rest condition with a mercury sphygmomanometer).The diagnose of hypertension was confirmed by a cardiologist.

We excluded from our study patients with history of stroke or transient ischemic attack; significant peripheral vascular disease (previous surgery or symptoms of intermittent claudication); aortic aneurysm; diabetes mellitus (fasting glucose > 126 mg/dl or receiving insulin or oral hypoglycemic treatment); thyroid disease; nephrotic syndrome or patients with prior coronary artery diseases (history of angina, previous myocardial infarction).

The characteristics of study population are shown in Table I.

Table I. Baseline characteristics

	Mean ± SD
Age	52±8 years
Systolic blood pressure	149±8 mm Hg
Diastolic blood pressure	96±5 mmHg
Men, %	382, 64%
Total cholesterol	216±72 mg%
LDLc	184±64 mg%
Smokers, %	420, 70%
Fasting glucose	86±12 mg%
Serum creatinine level	0.94 ±0.16 mg%

The study was approved by local Ethics Committee and informed consent was obtained from all participants.

Procedures

At the baseline examinations, blood pressure (BP) was measured three different times, using a single mercury sphygmomanometer with an appropriate cuff size, after participants had been seated for at least 5 minutes. Systolic and diastolic blood pressures (SBP and DBP) were recorded from the first and fifth Korotkoff sounds. Hypertensive subjects were divided into 3 groups: controlled (using medication, normal BP at examination), uncontrolled (using medication, elevated BP at examination), and untreated (elevated BP at examination, but not using medication). The complete history of cardiovascular diseases was taken and an ECG was performed and interpreted by a cardiologist; complete blood analysis (including fasting glycemia, serum creatinine, TSH, FT3, FT4) was done before inclusion in our study. The photographs were read in a masked manner by 2 trained graders, and the presence of any retinal changes was recorded.

All participants underwent a detailed ocular examination, after pupillary dilation, 30° stereoscopic retinal photographs of the macula, optic disc, and other retinal fields of both eyes were taken, using a fundus camera (model FF3; Carl Zeiss Meditech, Oberkochen, Germany).

We use the term hypertensive retinopathy to describe all retinal changes included in the Keith, Wagener, and Barker classification (Table II).

Table II. Keith, Wagener, and Baker classification of hypertensive retinopathy

Grade I	slight or modest narrowing of the retinal arterioles, with arteriovenous ratio ≥ 1:2
Grade II	modest to severe narrowing of retinal arterioles (focal or generalized), with arteriovenous ratio < 1:2 or arteriovenous nicking
Grade III	bilateral soft exudates or flame-shaped hemorrhages
Grade IV	bilateral optic nerve oedema

We have documented the following individual lesions as present versus absent: generalized and focal arteriolar narrowing, arteriovenous nicking, retinal hemorrhage and exudates, microaneurysms and disc swelling. Hypertensive retinopathy was defined to include the presence of any one of the above lesions.

The presence of coronary artery diseases was defined by history of pectoris angina (with one of the following: specific ischemic changes on ECG, positive effort test, positive coronarography) and also acute myocardial infarction (typical chest pain, specific rise and fall of cardiac enzyme and ST elevation on ECG). The diagnostic of coronary artery disease was made by a cardiologist from Cardiovascular Disease Institute Timisoara.

Statistical analysis

We used the MedCalc Software for calculating the relative risk, confidence interval and prevalence proportion. The results for continuous variables were given as mean ± SD and for categorical variables as percentage. The limit of P value of statistical significance was considered 0.05.

RESULTS

We included in our study 600 patients (382 men, 218 women), mean age 50 ± 12 years. The mean follow-up period was 26±12 months.

After initial evaluation of blood pressure 360 patients (60%) had first stage hypertension, 180 patients (30%) had second stage hypertension and 60 patients (10%) had third stage hypertension. No patient had malignant hypertension at initial evaluation.

We found hypertensive retinopathy in 210 patients (35%): focal and general arteriolar narrowing in 108 patients (18%); arteriovenous nicking in 60 patients (10%); microaneurysms, hemorrhages and exudates (retinopathy) in 42 patients (7%). No patient had disc swelling or optic nerve oedema. The characteristic lesions according to the type of hypertension are described in Table III.

Table III. Characteristic lesion according to hypertension type

Lesion			
Hypertension	Arteriolar narrowing	AV nicking	Retinopathy
Treated and controlled	72 (12%)	10 (1.6%)	3 (0.5%)
Treated and uncontrolled	26 (4.3%)	33 (5.5%)	12 (2%)
Untreated	10 (1.6%)	17 (2.83%)	27 (4.5%)

Retinopathy: microaneurysms, hemorrhages and/or exudates

During follow-up 150 patients were diagnosed with coronary artery disease (110 patients with stable angina, 24 patients with unstable angina, and 16 patients with acute ST elevation myocardial infarction). Out of them 100 patients had hypertensive retinopathy (38 patients retinopathy, 32 patients AV nicking and 30 patients arteriolar narrowing).

The presence of any retinopathy sign predicted a three fold higher risk for coronary artery disease (RR 3.71; 95% CI 2.76 to 4.98), with higher risk for patients with retinopathy (RR 4.19; 95% CI 3.41 to 5.15) as compared to AV nicking (RR 2.25; 95% CI 1.69 to 2.97) and arteriolar narrowing (RR 1.23; 95% CI 0.88 to 1.71).

The incidence of coronary artery disease was higher in patients with second and third stage hypertension, in patients with a poor control of blood pressure, despite treatment. Incidence of hypertensive retinopathy was higher in women, but the incidence of coronary artery disease was higher in men.

We didn't use in our study correlation with other risk factor such as smoking or high cholesterol level.

DISCUSSION

Poorly controlled systemic hypertension causes damage to the retinal microcirculation, so that recognition of hypertensive retinopathy may be important in cardiovascular risk stratification of hypertensive patients (16).

Several recent studies have shown that retinal microvascular changes can be reliably documented by retinal photographs. In general, reproducibility from photographs has been found to be excellent for well defined retinopathy signs (17-21).

Data from population-based studies indicate that hypertensive retinopathy signs, defined from retinal photographs, are seen in 3–14% of adult individuals aged ≥40 years (1, 19, 20, 21-26).

In our study we found hypertensive retinopathy in 210 patients (35%): focal and general arteriolar narrowing in 108 patients (18%); arteriovenous nicking in 60 patients (10%); microaneurysms, hemorrhages and exudates (retinopathy) in 42 patients (7%).

In the Beaver Dam Eye Study they find signs of retinopathy in 11% of subjects, arteriolar narrowing in 19% of subjects, and arteriovenous nicking in 3% of subjects in the nondiabetic population with systemic hypertension.

In two other population-based studies, both of which included subjects with diabetes, the relationship of blood pressure with retinopathy signs, arteriolar narrowing, and arteriovenous nicking was studied. Signs of retinopathy (exudates and flame-shaped hemorrhages) were detected with ophthalmoscopy in 0.8% of white males and 2.3% of white females, and arteriovenous nicking was detected in 11.5% of white males and 14.2% of white females in a population-based study in Evans County, Georgia (27). In that study, retinopathy was present in 2.3% of white males and 4.9% of white females, and arteriovenous nicking was present in 15.5% of white males and 23.2% of white females whose diastolic BP was greater than 100 mm Hg. In a population-based study of 855 men aged 50 years in Gothenburg, Sweden, in which both ophthalmoscopy and fundus photographs were used to detect retinopathy, hemorrhages were found in 0.4% of subjects, arteriolar narrowing in 6.0% of subjects, and arteriovenous nicking in 8.9% of subjects (28).

In our study the presence of hypertensive retinopathy was as detected using stereoscopic retinal photographs of the macula, optic disc, and other retinal fields of both eyes and interpreted by two experimented non-ophthalmologists. The incidence of retinopathy was higher in women, data similar with other study (23). Hypertensive retinopathy was associated with a three fold higher risk of coronary artery disease. We included in our study both men and women. Similar data are shown in a study that included 560 only men at high risk (29). Hypertensive retinopathy signs have been linked with both subclinical and clinical coronary heart disease and congestive heart failure. For example, various hypertensive retinopathy signs have been associated with ischemic changes on electrocardiogram (30), severity of coronary artery stenosis on angiography (31) and incident coronary heart disease and myocardial infarction in men (29) and women (32).

Low sensitivity of retinal abnormalities associated with hypertension indicates that hypertensive retinopathy is not common in hypertensive people. Less than half of the retinal changes associated with hypertension cannot be explained by high blood pressure (low positive predictive value). In both the Beaver Dam eye study and the Blue Mountains eye study little difference was found in the presence of hemorrhages and exudates between normotensive and hypertensive people aged over 65. Various other conditions have been associated with hypertensive retinopathy, such as ethnicity, smoking, intima-media thickness, carotid plaque score, carotid artery stiffness, serum cholesterol concentration, diabetes, and body mass index. The high specificity indicates that hypertensive retinopathy is rare in patients with normal blood pressure. Half the people without hypertensive retinopathy, however, still have hypertension (low negative predictive value).

A number of important limitations of this study should be mentioned. The people in our study were white Caucasians aged between 40 and 65 years. We didn't consider other important risk factors for coronary artery disease such as smoking, high cholesterol level, body mass index. Thus, caution should be taken when extending these findings to other segments of the population -older and younger age groups, other ethnic groups, etc.

CONCLUSION

Our study showed that evaluation of hypertensive retinopathy is important in hypertensive patients because the presence of any modification (arteriolar narrowing, arteriovenous nicking and retinopathy) is associated with three fold higher risk of developing coronary artery disease. The incidence of hypertensive retinopathy is higher in women, but still the men had a higher incidence of coronary artery disease. Patients with retinopathy (microaneurysms, hemorrhages and/or exudates) are at highest risk and it is important to have adequate control of blood pressure and other risk factors. Other studies are necessary to assess the correlation with cardiovascular risk factors, hypertensive retinopathy and cardiovascular mortality.

REFERENCES

1. Klein R, Klein BE, Moss SE, Wang Q. Hypertension and retinopathy, arteriolar narrowing, and arteriovenous nicking in a population. Arch Ophthalmol 1994; 112: 92-8.
2. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr. et al. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: the JNC 7 report. JAMA 2003; 289: 2560-72.
3. Cifkova R, Erdine S, Fagard R, Farsang C, Heagerty AM, Kiowski W et al Practice guidelines for primary care physicians: 2003 ESH/ESC hypertension guidelines. J Hypertens *2003*; 21: 1779-86.
4. Wong TY, Klein R, Klein BE, Tielsch JM, Hubbard L, Nieto FJ.Retinal microvascular abnormalities and their relationship with hypertension, cardiovascular disease, and mortality. *Surv Ophthalmol* 2001; 46**:** 59-80.
5. Tso MO, Jampol LM. Pathophysiology of hypertensive retinopathy. *Ophthalmol* 1982; 89**:** 1132–45.
6. Keith NM**,** Wagener HP, Barker NW. Some different types of essential hypertension: their course and prognosis. *Am J Med Sci* 1939;197:332-43.
7. Ralph RA**.** Prediction of cardiovascular status from arteriovenous crossing phenomena. *Ann Ophthalmol* 1974;6: 323-610.
8. Svardsudd K**,** Wedel H, Aurell E, *et al*. Hypertensive eye ground changes: prevalence, relation to blood pressure and prognostic importance. *Acta Med Scand* 1978;204: 159-67.
9. Palmer RS, Loofbourow D, Doering CR. Prognosis in essential hypertension: eight-year follow-up study of 430 patients on conventional medical treatment.N Engl Med J 1948;239:990–4.
10. Oglesby P, Lepper MH, Phelan WH, *et al*. A longitudinal study of coronary heart disease.Circulation 1963;28:20–31.
11. Breslin DJ**,** Gifford RW Jr, Fairbairn JF II, *et al*. Prognostic importance of ophthalmoscopic findings in essential hypertension. *JAMA* 1966;195:335-8.
12. Breslin DJ, Gifford RW Jr, Fairbairn JF II. Essential hypertension: a twenty year follow-up study. *Circulation* 1966;33:87-97.
13. Tanaka *H*, Hayashi M, Date C et al. Epidemiologic studies of stroke in Shibata, a Japanese provincial city: preliminary report on risk factors for cerebral infarction. *Stroke* 1997; 16, 773-80.
14. Nakayama T, Date C, Yokoyama T, Yoshiike N, Yamaguchi M, Tanaka H (*1997*) A 15.5-year follow-up study of stroke in a Japanese provincial city. The Shibata Study. *Stroke* 1997; *28*, *45*-52.
15. Wong TY, Klein R, Nieto FJ et al. Retinal microvascular abnormalities and ten-year cardiovascular mortality. A population-based case-control study. *Ophthalmology* 2003; 110, 933-40.
16. Chatterjee S, Chattopadhya S, Hope-Ross M, et al. Hypertension and the eye: changing perspectives. *J Human Hypertens* 2002;16: 667-75.
17. Sharrett AR, Hubbard LD, Cooper, et al. Retinal arteriolar diameters and elevated blood pressure. The Atherosclerosis Risk in Communities *Study.Am J Epidemiol* 1999;150:263-70.
18. Hubbard L, Brothers RJ, King WL, et al. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study. *Ophthalmology* 1999; 106:2269-80.
19. Klein R, Klein BEK, Moss SE. The relation of systemic hypertension to changes in the retinal vasculature: the Beaver Dam Eye Study. *Trans Am Ophthalmol Soc* 1997; 95: 329-48.
20. Wang JJ, Mitchell P, Leung H, et al. Hypertensive retinal wall signs in a general older population: the Blue Mountains Eye Study. *Hypertension* 2003; 42: 534-41.
21. Wong TY, Klein R, Klein BE, et al. Retinal vessel diameters and their associations with age and blood pressure. *Invest Ophthalmol Vis Sci* 2003; 44: 4644-50.
22. Klein R, Sharrett AR, Klein BEK et al. Are retinal arteriolar abnormalities related to atherosclerosis? The Atherosclerosis in Communities Study. *Arterioscler Thromb Vasc Biol*, 20, 1644-50.
23. Sharp PS, Chaturvedi N, Wormald R et al. Hypertensive retinopathy in Afro-Caribbeans and Europeans. Prevalence and risk factor relationships. *Hypertension* 1995; 25: 1322-5.
24. Stolk RP, Vingerling JR, de Jong PT et al. Retinopathy, glucose, and insulin in an elderly population. The Rotterdam Study. *Diabetes* 44: 11-15.
25. *Wong TY*, Klein R, Sharret AR et al. The prevalence and risk factors of retinal microvascular abnormalities in older people. The Cardiovascular Health Study. *Ophthalmology* 2003; 110: 658-66.
26. Yu T, Mitchell P, Berry G, Li W, Wang JJ. Retinopathy in older persons without diabetes and its relationship to hypertension. *Arch Ophthalmol* 1998; 116: 83-9.
27. McDonough JR, Garrison GE, Hames CG. Blood pressure and hypertensive disease among Negroes and whites: a study in Evans County, Georgia. *Ann Intern Med.* 1964; 61: 208-28.
28. Svardsudd K, Wedel H, Aurell E, Tibber G. Hypertensive eye ground changes:prevalence, relation to blood pressure and prognostic importance. *Acta Med Scand.* 1978; 204:159-167.
29. Duncan BB, Wong TY, Tyroler HA, Davis CE, Fuchs FD. Hypertensive retinopathy and incident coronary heart disease in high risk men. *Br J Ophthalmol.* 2002; 86(9): 1002-6.
30. Breslin DJ, Gifford RWJ, Fairbairn JFI. Essential hypertension: a twenty year follow-up study. Circulation 1996; 33: 87-97.
31. Michelson EL, Morganroth J, Nichols CW, MacVaugh H. Retinal arteriolar changes as an indicator of coronary artery disease. *Arch Intern Med* 1997: 139: 1139-41.
Wong TY, Klein R, Sharret AR et al. Retinal arteriolar narrowing and incident coronary heart disease in men and women: The Atherosclerosis Risk in the Communities Study. *JAMA* 2002; 287: 1153-9.

RELATIA DINTRE RETINOPATIA HIPERTENSIVA SI BOALA CORONARIANA

REZUMAT

Scopul studiului. Evidentierea relatiei dintre retinopatia diabetica si boala coronariana la populatia din judetul Timis, Romania.
Metode. Am efectuat un studiu observational care a inclus 600 de pacienti (382 barbati si 218 femei), cu varste cuprinse intre 40 si 60 de ani, care au fost examinati in vederea evidentierii modificarilor microvasculare prin fotografii retiniene, in cadrul Clinicii de Oftalmologie Timisoara, in perioada martie 2008, mai 2011. In studiul nostru au fost inclusi pacienti cu hipertensiune, pe baza valorilor tensiunii arteriale sistolice si diastolice determinate la 3 intervale succesive (valori mai mari de 140 mmHg ale TAS si/sau mai mari de 90 mmHg ale TAD, determinate in conditii de repaus, cu ajutorul unui sfigmomanometru cu mercur).
Rezultate. In urma evaluarii initiale a tensiunii arteriale, 360 de pacienti (60%) au prezentat hipertensiune arteriala stadiul I, 180 pacienti (30%) au prezentat HTA stadiul II, iar 60 de pacienti (10%) au fost detectati cu HTA stadiu III. La evaluarea initiala nici unul dintre pacienti nu a prezentat hipertensiune maligna.
Retinopatia diabetica a fost detectata la 210 pacienti (35%): ingustare arteriolara focala si generala la 108 pacienti (18%); constrictie arterio-venoasa la 60 de pacienti (10%); microanevrisme, hemoragii si exudate (retinopatie) la 42 de pacienti (7%). Nici unul dintre pacienti nu a prezentat exudate ale discului optic sau edeme ale nervului optic. Prezenta semnelor de retinopatie este predictiva pentru un risc de trei ori mai crecut de a dezvolta boala coronariana (RR 3,71; 95% CI 2,76 to 4,98), cu risc crescut pentru pacientii cu retinopatie (RR 4,19; 95% CI 3,41 to 5,15) comparativ cu constrictia AV (RR 2,25; 95% CI 1,69 to 2,97) si ingustarea arteriolara (RR 1,23; 95% CI 0,88 to 1,71). Incidenta bolii coronariene a fost mai crescuta la pacientii cu hipertensiune arteriala stadiile II si III, la pacientii cu control precar al tensiunii arteriale, in ciuda tratamentului recomandat. Incidenta retinopatiei hipertensive a fost mai crescuta in cazul pacientilor de sex feminin, in timp ce incidenta afectiunilor coronariene a fost mai crescuta pentru pacientii de sex masculin.
Concluzii. Studiul nostru demonstreaza ca evaluarea retinopatiei hipertensive este importanta in cazul pacientilor hipertensivi deoarece prezenta oricaror modificari (ingustare arteriolara, constrictie arterio-venoasa si retinopatie) este asociata cu un grad crescut de a dezvolta boala arteriala coronariana. Incidenta retinopatiei hipertensive este mai mare la persoanele de sex feminin, dar pacientii de sex masculin prezinta o incidenta mai crescuta a bolii coronariene. Pacientii cu retinopatie (microanevrisme, hemoragii si/sau exudate) prezinta un risc crescut. De aceea, este importanta efectuarea unui control adecvat al tensiunii arteriale si a altor factori de risc.

Cuvinte cheie: retinopatie hipertensiva, boala coronariana

REVIEW

POTENTIAL CIRCULATING MARKERS OF PREECLAMPSIA

DANA CARMEN ZAHA
Preclinical Department, University of Oradea, Faculty of Medicine and Pharmacy

ABSTRACT

Preeclampsia and eclampsia are estimated to be responsible up to 20% of maternal deaths per year. Preeclampsia is a multisystem disorder of pregnancy which is characterized by new onset hypertension and proteinuria that develop after 20 weeks of gestation in previously normotensive women and several other symptoms, such as edema, disturbance of hemostasis, renal or liver failure and the HELLP syndrome that may also complicate the clinical picture. The etiology remains insufficiently elucidated; a certainty is the central role played by the placenta in its pathology and the general consensus is that preeclampsia is an endothelial cell disorder. As research in the field of preeclampsia progresses, much of the attention in recent years has been focused on peptides related to angiogenesis. Recent data suggest that circulating factors that interfere with the action of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) play a major role in maternal manifestation of the disorder. Similarly, maternal plasma levels of PlGF have been shown to be significantly reduced in the second trimester in women who went to develop preeclampsia compared to controls. Many recent studies have therefore concentrated on factors which antagonize VEGF and PlGF to assess their role in the development of preeclampsia. Two of the most studied peptides, which are produced by the placenta, are soluble FMS-like tyrosine kinase (sFLT-1) and soluble endoglin.

This paper presents current knowledge on the biology of preeclampsia and review several biochemical markers which may be used to early diagnosis and monitor preeclampsia.

Key words: preeclampsia, markers, angiogenesis.

INTRODUCTION

Worldwide pregnancy-induced hypertension affects 10% of pregnancies and pre-eclampsia complicates about 5% of pregnancies; the range is 5-10% (1). Eclampsia occurs in about 1/2000 deliveries in resource-rich countries (2). In resource-poor countries, estimates of the incidence of eclampsia vary from 1/100 to 1/1700 (3,4). Although eclampsia is a rare complication of pregnancy, approximately 50,000 women worldwide are estimated to die annually because of eclampsia. The reported maternal mortality rate ranges from 1-20%. The perinatal mortality rate of neonates born to eclamptic mothers ranges from 1.3-3% (5).

Preeclampsia is a multi-system disorder of pregnancy, which is characterized by new onset hypertension (systolic and diastolic blood pressure of ≥ 140 and 90 mm Hg, respectively, on two occasions, at least 6 hours apart) and proteinuria (protein excretion of ≥ 300 mg in a 24 h urine collection or a dipstick ≥ 2+), that develop after 20 weeks of gestation in previously normotensive women. Edema, disturbance of hemostasis, renal or liver failure and the HELLP syndrome also complicate the clinical picture. Preeclampsia can show mild or severe symptoms and can have an early onset (preeclampsia starting before 34 weeks of gestation) or late onset (preeclampsia starting after 34 weeks of gestation). It can manifest as a maternal disorder only, with an appropriate fetal growing or it can present itself with a growth restricted fetus (in utero growth restriction IUGR) or sudden fetal distress. The maternal risks must be carefully weighed against the possible fetal benefits in temporizing management, as the risk of fatal deterioration of the maternal and/or fetal health condition is high.

Resolution of preeclampsia is the removal of the placenta and prematurity, with the adverse consequence of delivering a pre-term baby. Anyway, preeclampsia, with or without IUGR, remains a major cause of maternal and neonatal mortality and morbidity worldwide. Several prophylactic therapies like anti-oxidant vitamins, calcium or folic acid supplementation, Aspirin have so far failed to prove efficacious in the prevention of pre-eclampsia, although some benefit has been shown in high risk group: previous episodes of pre-eclampsia, obesity, black race, diabetes or insulin resistance, collagen vascular disease, thrombophilias, multiple gestation, molar pregnancy and extremes of age (<20 or >40 years) increase the risk for pre-eclampsia (6,7).

The etiology remains insufficiently elucidated; it is believed to be multifactorial and a certainty is the central role played by the placenta in its pathology. An imbalance between vasoconstrictor and vaso-relaxant factors was proposed as a possible cause of the hypertension accompanying pre-eclampsia. It was reported a relative decrease in the concentration of the circulating vasodilator angiotensin (1–7), compared with circulating levels of the vasoconstrictor angiotensin II (8). There also appears to be an increased sensitivity to Ang II in women with pre-eclampsia (9). The vasodilator prostaglandin I_2 is elevated in normal pregnancy, but its production is reduced in pre-eclampsia. The vasoconstrictor thromboxane A_2 is increased out of proportion to prostaglandin I_2 in pre-eclampsia but administration of aspirin, a thromboxane inhibitor, does not consistently prevent pre-eclampsia in pregnant women (10,11).

Received August 20th 2011. Accepted September 10th 2011. Address for correspondence: Dana Carmen Zaha, MD, PhD, Preclinical Department, Faculty of Medicine and Pharmacy, University of Oradea, 1 decembrie Square No. 10 Oradea, Romania, phone: +40723 604496, e-mail: danazaha@rdslink.ro

The general consensus is that preeclampsia is an endothelial cell disorder resulting in a microangiopathy of target organs such as brain, liver, kidney and placenta. Evidence to date suggests that oxidative stress, circulatory maladaptation, inflammation, humoral, mineral and metabolic abnormalities may all contribute to endothelial dysfunction and pathogenesis of preeclampsia (12,13,14,15). Recently, it was proposed instead that intrinsic failure in trophoblast differentiation, but the origin of preeclampsia might not be restricted to an alteration of trophoblast differentiation.

Endothelin, cellular fibronectin, plasminogen activator inhibitor-1and altered prostacyclin / thromboxane profile have been shown to be elevated in women who develop preeclampsia before they became symptomatic (16). Recent data suggest that circulating factors that interfere with the action of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) play a major role in this disorder. This is the why much of the attention in recent years has been focused on peptides related to angiogenesis.

ANGIOGENIC AND ANTIANGIOGENIC FACTORS

Angiogenesis represent the development of new blood vessels from existing endothelium and is essential for normal placental development. Angiogenesis requires the complex interplay between the pro-angiogenic factors like vascular endothelial growth factor VEGF and placental growth factor PlGF) with their receptors VEGF receptor-1 (VEGFR-1) and VEGFR-2 and these factors have been shown to be key components in regulating trophoblast cell survival and function (17, 18, 19, 20). As normal pregnancy progresses, maternal VEGF and PlGF circulate in high concentration (21). Placental levels of mRNA-encoding VEGF have been shown to be much lower in women with preeclampsia compared to controls and maternal plasma levels of PlGF have been shown to be significantly reduced in the second trimester in women who went on to develop preeclampsia compared to controls (22,23). The use of anti-VEGF antibodies for systemic treatment in cancer has shown a dose dependant association with hypertension and proteinuria (24), which may indicate that these factors have a role in the development of preeclampsia.

Many recent studies have concentrated on factors which antagonize VEGF and PlGF to assess their role in the development of preeclampsia. Two of the most extensively studied peptides, which are produced by the placenta, are soluble FMS-like tyrosine kinase (sFLT-1) and soluble endoglin.

Soluble FLT-1 (or soluble VEGF receptor 1 or sFLT-1) binds to and neutralizes the angiogenic actions of VEGF and PlGF and is thought to be one of the key peptides involved in the development of preeclampsia (25). Maternal serum levels of sFLT-1 have been shown to be elevated in women with preeclampsia compared to controls (26, 27), to correlate with disease severity (28) and to decrease markedly following delivery (26). Levels of sFLT-1 are increased in nulliparity when compared to multiparous women. Others researchers found that although fetal levels of sFLT-1, measured in cord blood are elevated in preeclampsia. Alterations in sflt-1 and PlGF are also more accentuated in early onset in comparison to late onset preeclampsia (29). It was also noticed that increased levels of sflt-1 were associated with IUGR (30).

Another peptide that has been implicated in the pathogenesis of pre-eclampsia is soluble endoglin. Soluble endoglin (Eng) is a co-receptor for transforming growth factor (TGF)-β1 and TGF-β3 that is highly expressed on cellular membranes of the vascular endothelium and on the syncytiotrophoblast. It functions as a modulator of TGF-β signaling and is involved in angiogenesis and the regulation of the vascular tone (31,32). The circulatory form of endoglin has been identified in normal pregnancy and in preeclampsia. In vitro, sEng acts as a negative regulator of angiogenesis, thereby impairing capillary formation by endothelial cells. Soluble Eng level rises up in preeclamptic patients compared to normotensive controls and its concentrations appear to grow higher with the severity of the symptoms and are the highest in preeclampsia complicated by the HELLP symptom (32,33). Pregnancies with IUGR without the maternal syndrome may also be particularized by high levels of sEng, suggesting that this factor is not specific for preeclampsia, but may be a marker for clinical conditions associated with an underlying placental pathology (34). However, these results remain conflicting as others demonstrated no association between IUGR and the levels of sEng. Another study has suggested that sEng may prove useful in differentiating preeclampsia from other hypertensive diseases of pregnancy, such as gestational or chronic hypertension. It had demonstrated that circulating sEng levels increase earlier and to a significantly greater degree in women who eventually develop pre-eclampsia. This process begins at 17–20 weeks in women who develop preterm pre-eclampsia and at 25–28 weeks in women who develop term pre-eclampsia (35,36).

It has therefore been proposed that the maternal endothelial dysfunction in preeclampsia was caused by the imbalance of the levels of circulatory angiogenic factors. Amounts of sflt-1 are apparent in second trimester-, but not first trimester blood, in women destined to develop preeclampsia, whereas PlGF and VEGF levels already show alterations at the end of the first trimester of pregnancy in these patients. In normotensive pregnancies, sflt-1 levels remain relatively stable until the last 2 months of gestation when they steadily increase. This increase is much more pronounced in pregnancies ending with preeclampsia and can discriminate this condition beginning approximately 5 to 8 weeks before the symptoms arise, in particular in cases with preterm (<37 weeks) symptoms. In contrary to sflt-1, the levels of circulatory PlGF increase gradually and peak at mid gestation before declining again in uneventful pregnancies. PlGF concentration profile follows a similar pattern in women who later developed preeclampsia, however with decreased amplitude. PlGF concentrations are already significantly reduced at the end of the first trimester and remain lower throughout pregnancy and the difference in circulatory PlGF between normotensive pregnancies and those affected by preeclampsia is the highest within weeks of the onset of the clinical symptoms. Urinary PlGF

is likewise lower in preeclamptic patients before and at the time of symptoms (37, 38). On the other hand, it has demonstrated that the angiogenic factor PlGF is reduced in trophoblast cells from tubal ectopic pregnancies when compared to that of intrauterine pregnancies. In addition, it shows that this difference can be assessed systemically and that serum PlGF levels are reduced in tubal ectopic pregnancy and miscarriage (39).

According to some studies, the presymptomatic alterations in sflt-1 levels appeared to be specific for preeclampsia as no changes are detected in women who later deliver SGA neonate or whose pregnancies are complicated by IUGR, compared to women with normal pregnancy outcome (35,37). However, others found that in a selected group of patients with abnormal uterine perfusion, similar alterations in sflt-1 and PlGF levels could be detected during the second trimester in cases with subsequent IUGR. Levels of sEng and sFLT-1 have been elevated in the serum of preeclamptic women at 17-20 weeks gestation when compared to controls. The level at 11-13 weeks was similar between cases and control (35). Both sEng and sFLT-1 appear to be important peptides in the pathogenesis of preeclampsia but when used alone, do not appear to have a sufficiently high positive predictive value to be translated into routine clinical practice.

Others studies have therefore evaluated the potential of combination between the pro- and anti-angiogenic factors PlGF and sflt-1 for the prediction of preeclampsia. The studies reported that the pattern of changes in the ratio of different combinations of these factors (PlGF/sEng; (sflt-1+sEng)/PlGF; etc), collected at 13 weeks and around 20 weeks, was more informative than the individual biomarkers at single time-point screening.

This highlights that the differential secretion of angiogenic molecules associated with embryo implantation could be used to define a diagnostic biomarker strategy.

CURRENT CANDIDATES FOR EARLIER DETECTION

Placental protein 13 (PP-13) is a small protein with 139 amino acids which is highly homologous (69%) to the human eosinophil Charcot-Leyden Crystal protein. The homodimer which is linked by disulfide bonds probably has special haemostatic and immunobiological functions at the feto-maternal interface or a developmental role in the placenta.PP-13 may interfere with the balance between vasoconstrictors and vaso-relaxants especially by interfering with arachidonic acid metabolites on tropfoblastic cells (42). Levels of PP-13 slowly increase during a normal pregnancy but abnormally low levels of PP-13 were detected in first trimester serum samples of women subsequently developing fetal growth restriction and preeclampsia (40-43). Elevated serum concentrations of PP-13 have been found in the second and third trimester in women with preeclampsia, IUGR and in preterm delivery (43). Other researchers demonstrated no statistical significance between early PP-13 levels in complicated pregnancies by SGA, IUGR.

The combination of several diagnostic tools results in improved predictive power as was shown by combined measuring of first trimester serum PP-13 levels and median uterine artery pulsatility index by ultrasound. This combination achieved a detection rate for preeclampsia of 90% with a false positive rate of 6% (45). However, this combination of serum PP-13 levels and uterine artery pulsatility index loses its predictive power when late second trimester (22–24 weeks of gestation) serum is analyzed (42).

Pentraxin 3 (PTX3, tumor necrosis factor stimulated gene-14) belongs to the same family as C-reactive protein (CRP) or serum amyloid P component and consists of 381 amino acids. PTX3 then interacts with several growth factors, extracellular matrix components and certain pathogens but is also involved in the activation of the complement system and facilitates pathogen recognition by phagocytes. It has been proposed as a marker of endothelial dysfunction and inflammation in pre-eclampsia. During pregnancy, PTX3 is increasingly expressed in amniotic epithelium, chorionic mesoderm, trophoblast terminal villi, and perivascular stroma of placentae. Some studies showed that in case of a future preeclampsia and IUGR the PTX3 plasma levels are even more increased in all three trimesters (45).

P-Selectin is a cell surface adhesion molecules expressed by platelets and endothelial cells upon activation and plays important roles in inflammatory reactions and in coagulation. P-selectin is rapidly shed from the cellular membrane of activated platelets and this release is suggested to contribute to most of the soluble isoform of the molecule that is found in the plasma (46). Preeclampsia is associated with extensive platelet activation (47-49). P-selectin-exposing microparticles with procoagulant activity, released from activated platelets, have been detected in the peripheral blood of preeclamptic women (50,51). In addition, soluble P-selectin has been repeatedly, though not constantly, observed in higher amounts in serum or plasma of patients with this disorder (52-55). It has recently been shown that alterations in the levels of soluble P-selectin before 20 weeks of gestation antedate the symptoms (56-58). This early up-regulation of soluble P-selectin has been suggested to reflect the early but still asymptomatic disturbances of the maternal vascular system. In one of these studies, P-selectin was identified as the marker with the highest discriminatory ability among three biomolecules evaluated between gestational weeks 11 to 15 (59). However, the combination of P-selectin with the two other markers showed a detection rate of only 59% (with a false-positive rate of 5%), which is not sufficient for a possible routine clinical implementation as a screening test.

PAPP-A (pappalysin 1, pregnancy-associated plasma protein A) is a disulfide bond linked homodimeric peptidase of 1628 amino acids and a mass of 400 kDa. It can be detected during pregnancy in maternal circulation mainly as a complex with the proform of the eosinophil major basic protein, an inhibitor of PAPP-A (60). Recent studies have shown that although reduced in all trimester serum levels of PAPP-A are associated with preeclampsia, levels are also low in other complications of pregnancy. It was described a small increase in likelihood ratio of developing preeclampsia with decreasing levels of PAPP-A. Although PAPP-A alone was not a good predictor for preeclampsia, they felt, similarly to PP-13, that sensitivity could be improved by combining with uterine artery Doppler studies (59).

CONCLUSION

Despite there exists many different potential markers for preeclampsia, the reliability of these markers in predicting preeclampsia has been inconsistent between different studies. Whereby preeclampsia is a multifaceted disorder, there is a need for high quality, large scale multi-center trials which enroll patients with different risks of developing the syndrome and throughout multi-ethnical background, in order to assess the predictive value of different markers and finally propose the best marker combination for a routine use in clinical settings.

Preeclampsia is an appropriate disease to screen and is possible to decreases both maternal mortality and perinatal mortality. Currently, the clinical value of an accurate predictive test for preeclampsia is not clear since we lack prevention. Intensive monitoring in women, who are at increased risk of developing preeclampsia, when identified by a predictive test, may lower the incidence of adverse outcome for both mother and the neonate. However, the effectiveness of such a strategy must be rigorously investigated. The implementation of clinical tests will require close collaboration between the medical institutions together with the pharmaceutical industry in order to develop functional and, as best as possible, affordable tests which could profit to the pregnant women worldwide. Further studies with large number of patients will be required to confirm these very promising preliminary results and assess the utility of analyzing these biomarkers in the clinical routine.

REFERENCES

1. WHO International Collaborative Study of Hypertensive Disorders of Pregnancy. Geographic variation in the incidence of hypertension in pregnancy. *Am J Obstet Gynecol* 1988; 158:80–83.

2.Douglas K, Redman C. Eclampsia in the United Kingdom. *BMJ* 1994; 309:1395–1400.

3.Crowther CA. Eclampsia at Harare maternity hospital. An epidemiological study. *S Afr Med J* 1985; 68:927–929.

4.Bergstrom S, Povey G, Songane F, et al: Seasonal incidence of eclampsia and its relationship to meteorological data in Mozambique. *J Perinat Med* 1992; 20:153–158.

5.WHO, 2004. Bethesda, MD. Global Burden of Disease for the Year 2001 by World Bank Region, for Use in Disease Control Priorities in Developing Countries, National Institutes of Health: WHO. Make every mother and child count. World Health Report, 2005, Geneva:World Health Organization, 2005. 2nd ed.

6.Karumanchi SA, Maynard SE, Stillman IE, et al: Preeclampsia: A renal perspective. *Kidney Int*. 2005; 67: 2101–2113.

7.Lain KY, Roberts JM. Contemporary concepts of the pathogenesis and management of preeclampsia *JAMA*. 2002; 287:3183–3186.

8.Brosnihan KB, Neves LA, Anton L, et al: Enhanced expression of Ang-(1–7) during pregnancy. *Braz J Med Biol Res*. 2004; 37:1255–1262.

9.AbdAlla S, Luther H, Massiery A, et al: Increased AT (1) receptor heterodimers in preeclampsia mediate enhance angiotensin II responsiveness. *Nat Med*. 2001;7: 1003–1009.

10.Mills JL, DerSimonian R, Raymond E, et al: Prostacyclin and thromboxane changes predating clinical onset of preeclampsia. *JAMA*. 1999; 282: 356–362.

11.Caritis S, Sibai B, Hauth J, et al: Low-dose aspirin to prevent preeclampsia in women at high risk. *N Engl J Med*. 1998; 338:701–705.

12.Hahn S, Gupta AK, Troeger C, et al: Disturbances in placental immunology: ready for therapeutic interventions? Springer Semin Immunopathol. 2006; 27:477–493.

13.Huppertz B. The feto-maternal interface: setting the stage for potential immune interactions. Semin Immunopathol. 2007; 29:83–94.

14.Jauniaux E, Poston L, Burton GJ. Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. *Hum Reprod Update*. 2006;12:747–755.

15.Hung TH, Skepper JN, Charnock-Jones DS, et al: Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. *Circ Res.* 2002; 90:1274–1281.

16.Taylor RN, de Groot CJ, Cho YK, et al: Circulating factors as markers and mediators of endothelial cell dysfunction in preeclampsia. *Semin Reprod Endocrinol.*1998; 16(1): 17-31.

17.Khaliq A, Li XF, Shams M, et al: Localisation of placenta growth factor (PlGF) in human term placenta. *Growth Factors*. 1996; 13:243–50.

18.Crocker IP, Strachan BK, Lash GE, et al: Vascular endothelial growth factor but not placental growth factor promotes trophoblast syncytialization in vitro. *J Soc Gynecol Investig*. 2001;8:341–346.

19.Desai J, Holt-Shore V, Torry RJ, et al: Signal transduction and biological function of placenta growth factor in primary human trophoblast. *Biol Reprod*. 1999; 60:887–892.

20.Shore VH, Wang TH, Wang CL, et al: Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. *Placenta*. 1997;18:657–665.

21.Ong S., Lash G., Baker P.N. Angiogenesis and placental growth in normal and compromised pregnancies. *Best Pract Res Clin Obstet Gynaecol*. 2000; 14:969–980.

22.Cooper J.C., Sharkey A.M., Charnock-Jones D.S. VEGF mRNA levels in placentae from pregnancies complicated by pre-eclampsia. *Br J Obstet Gynaecol*. 1996;103:1191–1196.

23.Kim S.Y., Ryu H.M., Yang J.H. Maternal serum and amniotic fluid inhibin A levels in women who subsequently develop severe preeclampsia. *J Korean Med Sci*. 2006; 21:452–456.

24.Ostendorf T., De Vriese A.S., Floege J. Renal side effects of anti-VEGF therapy in man: a new test system. *Nephrol Dial Transplant*. 2007; 22:2778–2780.

25.Venkatesha et al: Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature Med.* 2006;12:642–649.

26.Salahuddin S., Lee Y., Vadnais M. Diagnostic utility of soluble fms-like tyrosine kinase 1 and soluble endoglin in hypertensive diseases of pregnancy. *Am J Obstet Gynecol*. 2007;197:28.e1–28.e6.

27.Chaiworapongsa T, Romero R, Kim YM, et al: Plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated prior to the clinical diagnosis of pre-eclampsia. *J Matern Fetal Neonatal Med*. 2005;17:3–18.

28.Wolf M., Sandler L., Munoz K. First trimester insulin resistance and subsequent preeclampsia: A prospective study. *J Clin Endocrinol Metabol*. 2002;87:1563–1568.

29.Barton JR, Witlin AG, Sibai BM. Management of mild preeclampsia. *Clin Obstet Gynecol*. Sep 1999;42(3):455-69.

30.Stepan H, Kramer T, Faber R: Maternal plasma concentrations of soluble endoglin in pregnancies with intrauterine growth restriction. *J Clin Endocrinol Metab* 2007, 92:2831-2834.

31.St-Jacques S, Forte M, Lye SJ, Letarte M: Localization of endoglin, a transforming growth factor-beta binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. *Biol Reprod* 1994, 51:405-413.

32.Duff SE, Li C, Garland JM, et al: CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003, 17:984-992.

33.Jeyabalan A, McGonigal S, Gilmour C, et al: Circulating and placental endoglin concentrations in pregnancies complicated by intrauterine growth restriction and preeclampsia. *Placenta* 2008, 29:555-563.
34.Levine RJ, Maynard SE, Qian C, et al: Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*. 2004; 350:672–683.
35.Levine RJ, Lam C, Qian C, et al: Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med.* 2006; 355: 992–1005.
36.Rana S., Karumanchi S.A., Levine R.J. Sequential changes in antiangiogenic factors in early pregnancy and risk of developing preeclampsia. *Hypertension*. 2007;50:137–142.
37.Aggarwal PK, Jain V, Sakhuja V, et al: Low urinary placental growth factor is a marker of pre-eclampsia. *Kidney Int* 2006, 69:621-624.
38.Levine RJ, Thadhani R, Qian C, et al: Urinary placental growth factor and risk of preeclampsia. *JAMA* 2005, 293:77-85.
39.Nancy Fowler Larson, Novel Biomarker May More Quickly Confirm Ectopic Pregnancy, *J Clin Endocrinol Metabol*. November 3, 2010.
40.Huppertz B, Sammar M, Chefetz I, et al: Longitudinal determination of serum placental protein 13 during development of preeclampsia. *Fetal Diagn Ther* 2008, 24:230-236.
41.Chafetz I, Kuhnreich I, Sammar M, et al: First-trimester placental protein 13 screening for preeclampsia and intrauterine growth restriction. *Am J Obstet Gynecol* 2007, 197:35-37.
42.Spencer K, Cowans NJ, Chefetz I ,et al: First-trimester maternal serum PP-13, PAPP-A and second-trimester uterine artery Doppler pulsatility index as markers of preeclampsia. *Ultrasound Obstet Gynecol* 2007, 29:128-134.
43.Burger O, Pick E, Zwickel J, et al: Placental protein 13 (PP-13): effects on cultured trophoblasts, and its detection in human body fluids in normal and pathological pregnancies. *Placenta* 2004, 25:608-622.
44.Khalil A, Cowans NJ, Spencer K, et al: First trimester maternal serum placental protein 13 for the prediction of pre-eclampsia in women with a priori high risk. *Prenat Diagn* 2009
45.Nicolaides K.H., Bindra R., Turan O.M. A novel approach to first-trimester screening for early pre-eclampsia combining serum PP-13 and Doppler ultrasound. *Ultrasound Obstet Gynecol.* 2006;27:13–17.
46.Rovere-Querini P, Antonacci S, Dell'Antonio G, et al: Plasma and tissue expression of the long pentraxin 3 during normal pregnancy and preeclampsia.*Obstet Gynecol* 2006, 108:148-155.
47.Dunlop LC, Skinner MP, Bendall LJ, et al: Characterization of GMP 140 (P-selectin) as a circulating plasma protein. *J Exp Med* 1992, 175:1147-1150.
48.Holthe MR, Staff AC, Berge LN, Lyberg T. Different levels of platelet activation in preeclamptic, normotensive pregnant, and nonpregnant women. *Am J Obstet Gynecol* 2004, 190:1128-1134.
49.Konijnenberg A, Stokkers EW, Post JA van der, et al: Extensive platelet activation in preeclampsia compared with normal pregnancy: enhanced expression of cell adhesion molecules. *Am J Obstet Gynecol* 1997, 176:461-469.
50.Yoneyama Y, Suzuki S, Sawa R, et al: Plasma adenosine levels and P-selectin expression on platelets in preeclampsia. *Obstet Gynecol* 2001, 97:366-370.
51.Bretelle F, Sabatier F, Desprez D, et al: Circulating microparticles: a marker of procoagulant state in normal pregnancy and pregnancy complicated by preeclampsia or intrauterine growth restriction. *Thromb Haemost* 2003, 89:486-492.
52.Lok CA, Nieuwland R, Sturk A, et al: Microparticle-associated P-selectin reflects platelet activation in preeclampsia. *Platelets* 2007, 18:68-72.
53.Aksoy H, Kumtepe Y, Akcay F, et al: Correlation of P selectin and lipoprotein(a), and other lipid parameters in preeclampsia. *Clin Exp Med* 2002, 2:39-43.
54.Chaiworapongsa T, Romero R, Yoshimatsu J, et al: Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. *J Matern Fetal Neonatal Med* 2002, 12:19-27.
55.Halim A, Kanayama N, Nakashima A, et al: Plasma P selectin (GMP-140) and glycocalicin are elevated in preeclampsia and eclampsia: their significances. *Am J Obstet Gynecol* 1996, 174:272-277.
56.Heyl W, Handt S, Reister F, et al: Elevated soluble adhesion molecules in women with pre-eclampsia. Do cytokines like tumour necrosis factor alpha and interleukin-1beta cause endothelial activation. *Eur J Obstet Gynecol Reprod Biol* 1999, 86:35-41.
57.Banzola I, Farina A, Concu M, et al: Performance of a panel of maternal serum markers in predicting preeclampsia at 11–15 weeks' gestation. *Prenat Diagn* 2007, 27:1005-1010.
58.Bosio PM, Cannon S, McKenna PJ, O'Herlihy C, et al: Plasma P-selectin is elevated in the first trimester in women who subsequently develop pre-eclampsia. *BJOG* 2001, 108:709-715.
59.Chavarria ME, Lara-Gonzalez L, Garcia-Paleta Y, et al: Adhesion molecules changes at 20 gestation weeks in pregnancies complicated by preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 2008, 137:157-164.
60. Spencer K., Cowans N.J., Nicolaides K.H. Low levels of maternal serum PAPP-A in the first trimester and the risk of pre-eclampsia. *Prenat Diagn*. 2008;28:7–10.

MARKERI POTENTIALI CIRCULANTI AI PREECLAMPSIEI

REZUMAT

Preeclampsia şi eclampsia sunt estimate a fi responsabile pentru până la 20% din decesele materne anuale. Preeclampsia este o tulburare specifică sarcinii caracterizată prin hipertensiune şi proteinurie care se dezvoltă după săptămâna 20-a de sarcină la femei normotensive anterior. Edemul, tulburările hemostazei, ale funcţiilor renale şi hepatice, precum şi sindromul Hellp pot complica tabloul clinic. Etiologia rămâne insuficient cunoscută; cert este rolul central pe care îl joacă placenta, iar consensul general este că, preeclampsia este o tulburare a celulelor endoteliale. Progresele în cercetările asupra preeclampsiei au fost în ultimii ani mai ales îndreptate asupra peptidelor implicate în angiogeneză. Date recente sugerează că, factori circulanţi care interferă cu acţiunea factorului de creştere endothelial (VEGF) şi factorul de creştere placental (PlGF) joacă un rol important în manifestarea maternă a acestei tulburări. Astfel, nivelul plasmatic matern al PlGF a fost semnificativ redus în al doilea trimestru la femei care au dezvoltat preeclampsie comparativ cu grupul de control. Alte studii recente şi-au concentrat interesul asupra factorilor care se opun acţiunii VEGF şi PlGF pentru a determina rolul lor în dezvoltarea preeclampsiei. Două dintre cele mai studiate asemenea peptide produse de placentă sunt: tirozin kinaza FMS-like solubilă (sFLT-1) şi endoglina solubilă.

Acest articol îsi propune să prezinte cunostintele de actualitate referitoare la biologia preeclampsiei si revizuirea unor markeri biochimici care pot fi utilizati în diagnosticul precoce si monitorizarea preeclampsiei.

Cuvinte cheie: preeclampsia, markeri, angiogeneza

NEW APPROACHES FOR THE DRIED BLOOD SPOT ANALYSIS

ILEANA SCURTU[1], ALEXANDRA GRUIA[2], VALENTIN ORDODI[1, 2], ELENA CIURARIU[1], FELIX MIC[2], CALIN TATU[1, 2], VICTOR DUMITRASCU[1, 2]1

[1]Victor Babes University of Medicine and Pharmacy, Timisoara

[2] Emergency Clinical County Hospital, Timisoara

ABSTRACT

The recent recognition of dried blood spot (DBS) technology, as an alternative to plasma as the matrix for systemic measurement of drug analytes in pharmacokinetic (PK) and toxicokinetic (TK) studies. This article is a review of various types of analysis of dried blood spot, from off-line extraction to on-line extraction, and various applications in areas as genetics, virology, pharmacology, toxicology. This technique permits the decrease of blood volume which is necessary to analysis, making possible pharmacokinetics studies on small lab animals. In the last few years new concepts of extraction have emerged, thus there is developed an in line extraction method of pharmaceutical compounds from dried blood spot prior chromatography analysis.

Keys words: dried blood spot, paper DBS**,** pharmacokinetic, toxicology

INTRODUCTION

Dried blood spot (DBS) analysis is a blood samples preparation technique by liquid-solid extraction. The preparation of samples is one of the most important parts of the analytical process, because influences the detection, the repeatability, and the reproducibility of the analysis.

This technique of dried blood spot (DBS) was first described in 1913 by Bang (1) in the estimation of blood glucose concentration. However, the use of dried blood spots dates to the early 1960s when Dr. Robert Guthrie developed an assay for the detection of phenylketonuria (2). His application of collecting blood on filter paper led to the population screening of newborns for the detection of inherited metabolic diseases. In recent years, the collection of blood on filter paper has become a significant tool for screening individuals for clinical purposes, for animal testing for preclinical purposes, for therapeutic and illicit drug monitoring, and for the application to routine drug development. Nowadays, pressure in the pharmaceutical industry to deliver high-quality pharmacokinetic data in the shortest possible timeframe is requiring workers in the field to accurately and precisely collect, share, store, and analyze thousands of biological samples. In addition the movement to reduce, refine, and replace the use of animals in drug development is challenging analysts to collect adequate samples while remaining within acceptable total blood collection volumes and avoiding excess animal usage. Thus DBS analysis is a way to address these challenges in the pharmaceutical industry. In addition, the use of techniques picked up in performing DBS analysis is extending this approach to other fields.

Recent advances such as the production of monoclonal antibodies, expression of synthetic proteins, and the introduction of the polymerase chain reaction have overcome many of these problems. This type of blood testing is now available for uses at home by consumers in the U.S. Available blood tests include Vitamin D, estrogen, testosterone, cortisol, TSH and lipids. New York is the only state that prohibits home blood spot testing (3)

1. Papers for DBS analysis

Schleicher & Scheull 903 (S&S 903, Whatman 903 or 903®) paper (**Fig.1**), manufactured from 100% pure cotton linters with no wet-strength additives added, is an FDA listed class II medical device.

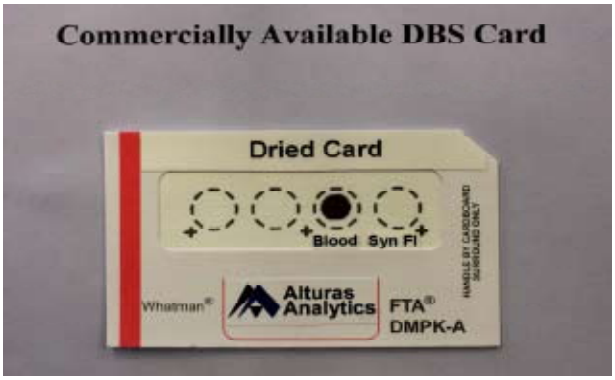

Fig.1. Commercially Available DBS card

The paper has been extensively used in newborn screening and other applications worldwide. Some other cards/papers, e.g. no. 545 paper (Advantec, Toyo, Tokyo), have also been used. The DBS paper/card might be further treated *via* impregnation for an improved extraction recovery (van der Heijden et al., 2009). In addition to particle retention, pore size and thickness that determine the loading capacity and spreadability of blood sample onto the DBS paper/card, uniformity and absorption characteristics of the

Received June 24th 2011. Accepted September 1st 2011. Address for correspondence: Ileana Scurtu, PhD, "Victor Babes" University of Medicine and Pharmacy Timisoara, Eftimie Murgu Square No. 2A, 300041, Timisoara, Romania, phone/fax: +40256490507, e-mail: ileana.scurtu@gmail.com

card/paper from lot to lot are important. In neonatal screening, the performance of DBS paper is monitored by the Newborn Screening Quality Assurance Program (NSQAP) to ensure that any new paper lots are consistent with the established guidelines and that performance is consistent from lot to lot. NSQAP has developed a procedure to evaluate the paper's uniformity and absorption characteristics. The procedure involves mixing washed red blood cells with plasma or serum to give an adjusted hematocrit of 0.55±0.01. The 'whole blood' is then enriched with 125I-l-thyroxine, and a 100 µL volume of the blood is applied onto the paper lots to be tested. The absorption time and the diameter of spots are then measured, and the blood volume for 3.2 mm disks is determined by measuring and comparing the total counts using a gamma counter. FTA and FTA Elute cards, also manufactured by Whatman (now part of GE Healthcare), are designed for nucleic acid analysis.

According to the manufacturer, both cards are chemically treated with proprietary reagents that, upon contact, lyses cells, denature proteins and prevent the growth of bacteria and other microorganisms. Recently, both cards have been employed in combination with LC-MS/MS for the quantitative analysis of small drug molecules and their metabolites in preclinical and clinical studies (4).

2. DBS sample collection

Two methods are commonly used to obtain capillary whole blood on a filter paper. The drop of blood can be collected directly on the filter paper or with the aid of a precision capillary. As the complete drop can be excised instead of punching out a disk from it, the influence of sample volume, hematocrit and sampling technique is minimized.

In the procedure to obtain a DBS, the hand is first cleaned and held down or warmed for a few minutes. With the help of an automatic lancet, the fingertip is pricked. While the first drop is wiped off with a sterile piece of cloth because of the presence of tissue fluid, the following drops are collected in a 50-µl precision capillary. Then, once completely filled, the entire capillary is placed in the centre of two concentric circles pre-printed on a Whatman 903 filter paper. The inner circle (10 mm diameter) must be entirely filled with blood, but blood may not pass the outer circle line (15 mm diameter), which was used for excision of the DBS. Although the blood is spot on just one side, both sides of the filter paper must be coloured. After visual inspection of the DBS analyze can start. The DBS are dried for minimum 4 hours at ambient temperature and subsequently analyzed or preserved in a sealable plastic bag at room temperature or -20 °C until analysis (**Fig.2**) (5).

Fig. 2. DBS blood sample collection technique

3. Drying, Storage, Transportation and Punching

It is very important to dry blood spots completely before storage or transportation. In order to be transported DBS samples are kept in plastic bags containing desiccants and a humidity indicator and portable refrigerators at -20°C. However, the drying time depends on the type of paper/card and the blood volume applied. In general, DBS samples are drying 2-3 h at room temperature, or room temperature 3-4 hours in horizontal position, dried at room temperature and stored at 4°C in zip-lock poly bags (**Fig.3.a** and **3.b**). However, samples that contain unstable compounds should be stored at a lower temperature (e.g. 2–8°C, ≤−15°C or ≤−60°C) in order to enhance stability. DBS samples that have been packed as described above can be transported through the mail in a high-quality bond envelope (6).

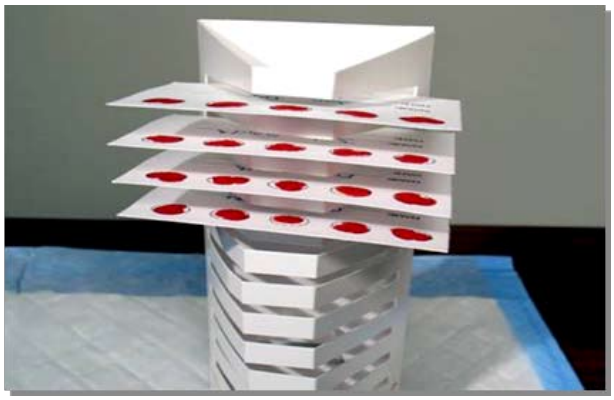

Fig. 3a. Drying of DBS samples

Fig. 3b. Storage of DBS samples

Manual punching can be a laborious and repetitive process. Typical punch size is 3–4 mm, but available sizes range from 0.35 to 8 mm. Punch size can be optimized based on the required limit of detection (i.e., a larger punch size leads to an increased signal) and available spotted blood volume. This can result in hand and thumb fatigue, especially when a large number of spots are punched. In an attempt to alleviate this, **GSK–Research Triangle Park** developed the prototype automated hand puncher (Figure 3), which incorporates a spinning core punch that helps to cut the DBS disk from the paper. It has been shown to decrease hand fatigue, due to less external force needed to punch the disk,

but still has the same time constraints as a single punch. Manual punch times typically vary by analyst, but are within 10–15 min per 96 samples, enables clot detection, and a proper liquid sensing program are critical for accurate pipetting (6).

4. DBS sample processing and analysis

Five microliters of real or spiked whole blood were spotted on a filter paper card using a volumetric micropipette. The blood spots are allowed to dry at room temperature for 2 h and then packed in a sealable plastic bag containing desiccant until analysis. They were stored in the dark at ambient temperature except for short-term stability experiments, in which a variety of temperatures were tested. Before analysis, a disc (i.d. 10 mm) containing the whole DBS was manually punched out and directly introduced into the desired position of the DBS prototype (**Fig.4**). Then, a volume of internal standard was added to the disc using the same volumetric micropipette used above (7).

On-line desorption of dried blood spots (on-line DBS) (**Fig.4**), allowing the direct analysis of a dried blood spot coupled to liquid chromatography mass spectrometry device. The system is based on an inox cell which can receive a blood sample (10 µL) previously spotted on a filter paper. The cell is integrated into LC/MS system where the analytes are desorbed out of the paper towards a column switching system ensuring the purification and separation of the compounds before their detection on a single quadrupole MS coupled to atmospheric pressure chemical ionisation source. The described procedure implies that no pretreatment is necessary in spite the analysis is based on whole blood sample. To ensure the applicability of the concept couple of drugs were chosen. Despite the use of a small sampling volume and a single quadrupole detector, on-line DBS allowed the analyses of the compounds over their therapeutic concentrations. The method showed good repeatability with relative standard deviation (RSD) lower than 15% based on two levels of concentration (low and high). Function responses were found to be linear over the therapeutic concentration for each compound and were used to determine the concentrations of real patient samples. Comparison of the founded values with those of a validated method used routinely in a reference laboratory showed a good correlation between the two methods. Good selectivity was observed ensuring that no endogenous or chemical components interfered with the quantitation of the analytes. This work demonstrates the feasibility and applicability of the on-line DBS procedure for bioanalysis (7).

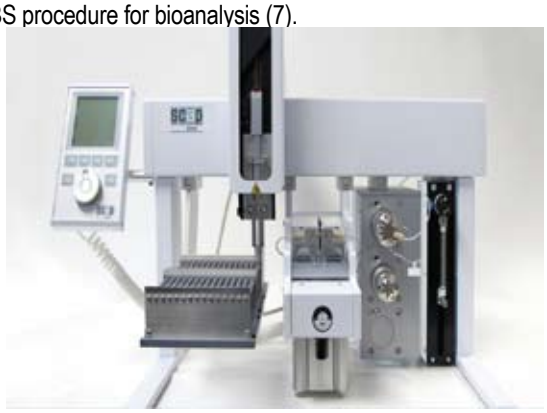

Fig. 4. On-line DBS prototype

5. Applications

a) Genetics

The stability of genomic DNA stored in dried blood specimens is a major concern for genetic studies. The dried blood specimens have been retained for a long period based on the stalability of DNA is the most stable analyte in dried blood and an important tool for population-based genetic studies. Casole and colleages showed that the maximum length of stability of HIV proviral DNA in dried blood spots kept at ambient temperature and dessicated at -20ºC was about 3.5 months. Beta-globin DNA has been detected in dried blood stored at ambient temperature for about 1 year (8).

b) Virology

Quantification of HIV-RNA from dried blood spots (DBS) has been evaluated in several studies using different methodologies (9,10). Overall, the results have shown that determination of viral load in DBS is well correlated to plasma levels, especially when HIV-RNA is above a certain threshold (generally ~3.5 log copies/mL), and its clinical utility to detect virological failure in resource-limited settings has been confirmed (11). Particularly good results have been obtained with new methodologies for HIV-RNA quantification based on real-time PCR technology (12,13.14).

Previous studies (9,14) have suggested that the presence of proviral HIV-DNA could affect the results obtained from DBS. Viljoen et al.(14), testing DBS with and without DNase pretreatment, concluded that a prior DNA treatment step was a prerequisite for accurate monitoring. In other study was found that ~80% of the HIV-RNA values obtained from DBS were lower than those obtained from plasma, although this proportion was lower in samples with <5000 copies/mL, suggesting that the possible DNA contamination may play a role in samples with low levels of HIV-RNA. Some results suggested that a further step in DBS processing could be avoided in order to make the procedure easier and feasible in resource-limited settings.

Other previous studies have evaluated the stability over time of DBS for viral load quantification. Some of them (9,12,14) have shown significant stability over 3–12 months of storage at room temperature. Others (16,17) have suggested slight to significant decreases. In other studies (18), 7 months of storage at room temperature did actually cause a change in quantification, with a significantly higher mean difference between plasma and DBS HIV-RNA values. The most important differences were observed for the lowest viral loads (and indeed a higher variability for low copy samples was also observed in the first series), suggesting that the use of DBS stored for several months, although not recommended, could still be considered in specific circumstances to detect virological failure (17).

In conclusion, HIV-RNA quantified from DBS was strongly correlated with that measured in plasma. The detection rate in DBS was 100% when the plasma level was ≥3.0 log. Quantification of HIV-RNA from DBS by the VERSANT assay can be used to diagnose virological failure in patients with HIV (17).

c) Newborn screening

Newborn screening refers to the process where babies are given a simple blood test a few days after birth to see if they have a rare genetic or metabolic condition. The conditions screened for in Newborn Screening (NBS) may be life threatening and/or cause intellectual disability.

These conditions are often referred to as 'inborn errors of metabolism'.

- Metabolism is the chemical process by which food is broken down to make energy available for the normal functioning, growth, and development of the body;

- Enzymes are proteins that are used by the cells to break down food into a form that can be used;

- Errors in metabolism occur when the essential enzymes are absent or malfunction;

The aim of NBS is to detect the conditions before the onset of symptoms so treatment can be started early to reduce the effect of the condition.

This form of testing is known as *screening* because it involves testing a whole population - in this case, newborn babies. All babies are tested even if they do not have any obvious signs of a condition that affects their metabolism.

Over 30 rare conditions can now be detected by the newborn screening test. Such early detection enables treatment to be undertaken as soon as possible if a baby is shown to have the condition for which testing is done (7).

d) Toxicology

DBS technique is used also in toxicology field. Analytes measured in toxicological applications of DBS are: elements (e.g. Pb); environmental pollutants; therapeutic drugs; drugs of abuse (e.g. phosphatidyl-ethanol, cotinine, benzodiazepines, morphine, methadone, fentanyl, cocaine, gamma-hydroxybutyric acid, etc.). These compounds should be derivatized "on spot" in order to be LC/MS, LC-MS/MS or GC/MS analyzed (7).

e) Pharmacokinetics

For drugs showing little variability in the fraction unbound in plasma (fu) and the blood cell-unbound plasma concentration ratio (ρ) under conditions of study, there is little concern in the use of DBS as an alternative to plasma. However, caution should be exercised when variability in either fu or ρ is large. Then it is important to identify, quantify, and, where possible, correct for, or accommodate, the variability, especially when interpreting pharmacokinetics data or linking pharmacokinetics to pharmacodynamics. For drugs with a blood-to-plasma ratio approaching the lower limit of 0.55, indicating minimal blood cell uptake, general concern is with variability in fu (about which much is known) when using DBS measurement as a surrogate for unbound drug in plasma. For drugs with larger values of the blood-to-plasma ratio, especially >2, variability in blood cell affinity becomes the critical factor. Fortunately, the magnitude and determinants of much of the variability can be evaluated in vitro using blood obtained from the study population of interest, which should be undertaken prior to implementing the use of DBS in a drug development program. So too should the study of the influence of drug concentration, temperature, and kinetics of blood cell distribution (1).

6. Advantages and Disadvantages of DBS

There are some advantages of DBS technique:

• Small volume samples: great for pediatric studies; use one animal in preclinical rodent studies for multiple draws – improves reproducibility, and reduces cost;

• Uses besides whole blood, also urine, synovial fluid, saliva, lavage fluid, tears;

• Easier patient recruitment – finger prick not venous draw;

• Ideal for Phase II/III where collection and storage of samples at non-ambient conditions not possible – no freezers, no centrifuges, no thawing;

• Advantages for ill patients (e.g. oncology);

• Shipment of samples on cards deactivates HIV, HCV, etc.

• Compound stability is better than in solution phase.

There are some disadvantages of DBS technique:

• Uses blood as matrix for exposure and pharmacokinetic determinations – conventionally, plasma used for ease of handling, shipping, storing; FDA states blood is an acceptable matrix for exposure and pharmacokinetic;

• Not a pre-concentration technique – LLOQ of 1-10 ng/mL typical (19);

FUTURE DIRECTION

Interest in DBS technology has gained momentum in the past several years. Areas of improvement in the field of DBS analysis include increased automation, ability to spray-deposit internal standard on the DBS card, specialty papers that include stabilizing and/or derivatization reagents, and direct analysis without the need for sample preparation (20).

CONCLUSIONS

DBS as a blood sampling technique for applications in drug development is a recent phenomenon, spurred by financial and ethical motivations. It is remarkable that the power of modern analytical technology has allowed an old sampling technique to become prominent in a short period of time. In view of its advantages compared to traditional plasma-based strategies.

Expansion of dried spot sampling involves developing new analytical strategies to be competitive with high-throughput requirements. As described, the prototype presents novel opportunities in filter paper analysis by affording an efficient tool for rapid and direct analyses of DBS specimens.

Automation and technological advances will help further the implementation of DBS methods in the laboratory. While the benefits of executing this technology in regard to the 3Rs (refinement, reduction, and replacement) are well understood and documented, development and analysis of DBS methods are still tedious and time consuming. Future automation and direct analysis from the DBS can expedite this procedure.

REFERENCES

1. Rowland M, Emmons GT. Use of Dried Blood Spots in Drug Development: Pharmacokinetic Considerations, *The AAPS Journal*, 2010; 12(3):290-293.
2. Guthrie R and Susi A. *Pediatrics*, 1963; 32: 338-343.
3. Parker SP, Cubitt WD. The use of the dried blood spot sample in epidemiological studies. *J. Clin. Pathol.*, 1999; 52 (9): 633-9.
4. http://www.whatman.com
5. Ingels A-S ME, Lambert WE, Stove CP. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal Bioanal Chem*, 2010; 398:2173-2182.
6. Craft NE, Haitema T, Brindle LK, Yamini S, Humphrey JH, West KP Jr. Retinol Analysis in Dried Blood Spots by HPLC. *J.Nutr.*, 2000; 130: 882-885.
7. Déglon J, Thomas A, Cataldo A, Mangin P, Staub C. On-line desorption of dried blood spot: A novel approach for the direct LC/MS analysis of micro-whole blood samples. *J Pharm Biomed Anal.*, 2009; 49(4):1034-9.
8. Chaisomchit S, Wichajarn R, Janejai N, Chareonsiriwatana W. Stability of Genomic DNA in Dried Blood Spots Stored on Filter Paper, *Southeast Asian J Trop Med Public Health*, 2005; 36(1): 270-3.
9. Brambilla D, Jenning C, Aldrovrandi G et al. Multicenter evaluation of use of dried blood and plasma spot specimens in quantitative assays for human immunodeficiency virus RNA: measurement, precision, and RNA stability. *J Clin Microbiol* 2003; 41: 1888-93.
10. Waters L, Kambugu A, Tibenderana H et al. Evaluation of filter paper transfer of whole-blood and plasma samples for quantifying HIV RNA in subjects on antiretroviral therapy in Uganda. *J Acquir Immune Defic Syndr,* 2007; 46: 590-3.
11. Johannessen A, Garrido C, Zahonero N et al. Dried blood spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania. *Clin Infect Dis*, 2009; 49: 976-81.
12. Mbida AD, Sosso S, Fleri P et al. Measure of viral load by using the Abbott Real-Time HIV-1 assay on dried blood and plasma spot specimens collected in 2 rural dispensaries in Cameroon. *J Acquir Immune Defic Syndr* 2009; 52: 9-16.
13. Andreotti M, Pirillo M, Guidotti G et al. Correlation between HIV-1 viral load quantification in plasma, dried blood spots, and dried plasma spots using the Roche COBAS Taqman assay. *J Clin Virol*, 2010; 1: 4-7.
14. van Deursen P, Oosterlaken T, Andre P et al. Measuring human immunodeficiency virus type 1 RNA loads in dried blood spot specimens using NucliSENS EasyQ HIV-1 v2.0. *J Clin Virol*, 2010; 47: 120-5.
15. Viljoen J, Gampini S, Danaviah S et al. Dried blood spot HIV-1 RNA quantification using open real-time systems in South Africa and Burkina Faso. *J Acquir Immune Defic Syndr*, 2010; 55: 290-8.
16. Leelawiwat W, Young NL, Chaowanachan T et al. Dried blood spots for the diagnosis and quantitation of HIV-1: stability studies and evaluation of sensitivity and specificity for the diagnosis of infant HIV-1 infection in Thailand. *J Virol Methods*, 2009; 155: 109-17.
17. Monleau M, Butel C, Delaporte E et al. Effect of storage conditions of dried plasma and blood spots in HIV-1 RNA quantification and PCR amplification for drug resistance genotyping. *J Antimicrob Chemother*, 2010; 65: 1582-6.
18. Pirillo MF, Recordon-Pinson P, Andreotti M, Mancini MG, Amici R, Giuliano M. Quantification of HIV-RNA from dried blood spots using the Siemens VERSANTw HIV-1 RNA (kPCR) assay, *J Antimicrob Chemother*, 2011; 66(12): 2823-6.
19. Wiseman JM, Evans CA, Bowen CL, Kennedy JH. Direct analysis of dried blood spots utilizing desorption electrospray ionization (DESI) mass spectrometry. *Analyst*, 2010; 135: 720-5.
20. Abu-Rabie P, Spooner N. Direct quantitative bioanalysis of drugs in dried blood spot samples using a thin-layer chromatography mass spectrometer interface. *Anal. Chem.*, 2009; 81(10): 275-84.

NOI ABORDARI PENTRU ANALIZA SPOTULUI DE SANGE USCAT

REZUMAT

Tehnica spotului de sange uscat (DBS) este recunoscuta recent ca o metoda alternativa la analiza medicamentelor (analitilor) din plasma in studii de farmacocinetica si toxicocinetica. Acest articol reprezinta un review despre diversele tipuri de analiza a picaturilor de sange uscat, pornind de la extractia off-line la cea on-line, precum si diversele sale aplicatii in domenii ca genetica, virusologie, farmacologie, toxicologie. Aceasta tehnica permite micsorarea volumului de sange necesar pentru analiza, facand astfel posibile studiile de farmacocinetica pe animale mici de laborator. In ultimii ani s-au dezvoltat noi concepte de extractie, astfel s-a dezvoltat o metoda de extractie in line pentru analiza compusilor farmaceutici din picaturile de sange uscat anterior unei analize cromatografice.
Cuvinte cheie : spotul de sange uscat, hartia DBS, farmacocinetica, toxicologie

Physiology Department
"Victor Babes" University of
Medicine and Pharmacy Timisoara

Romanian Society of Physiological Sciences

PRELIMINARY ANNOUNCEMENT

Physiology of the XXIst century – from genotype to phenotype

The 11th National Congress of Romanian Society of Physiological Sciences
Timisoara, 10-12 May 2012

ORGANIZING COMMITTEE

President: Prof. Dr. Carmen Panaitescu

Secretaries: Asist. Univ. Dr. Daciana Nistor, Philologist Carmen Pitic

Prof. Dr. Smaranda Goția
Conf. Dr. Gabriela Tănasie
Ș.l. Dr. Carmen Tatu
Ș.l. Dr. Lavinia Noveanu
Ș.l. Dr. Bojin Florina

Asist.Univ. Dr. Daniela Crîsnic
Asist.Univ. Dr. Adriana Gherbon
Asist.Univ. Dr. Laura Smaranda Goția
Asist.Univ. Dr. Meda Deică
Asist.Univ. Dr. Sabine Groza

SCIENTIFIC COMMITTEE

Prof. Dr. Mureşan Adriana (UMF Iuliu Haţieganu Cluj Napoca)
Preşedinte Societatea Română de Ştiinţe Fiziologice
Prof. Dr. Dobreanu Dan (UMF Tg. Mureş)
Prof. Dr. Gusti Simona (UMF Craiova)
Prof. Dr. Iancău Maria (UMF Craiova)
Prof.Dr. Ion Ileana (UMF Ovidius Constanţa)
Prof. Dr. Panaitescu Carmen (UMF Victor Babeş Timişoara)
Prof. Dr. Păunescu Virgil (UMF Victor Babeş Timişoara)
Prof. Dr. Sabău Marius (UMF Tg. Mureş)
Prof. Dr. Saulea Aurel (USMF Chişinău)
Prof. Dr. Schneider Francisc (UMF Arad)
Prof. Dr. Schneider Francisc (UMF Arad)

Prof. Dr. Zăgrean Leon (UMF Carol Davila Bucureşti)
Conf. Dr. Bădărău Anca (UMF Carol Davila Bucureşti)
Conf. Dr. Cismaş Petrişor (UMF Oradea)
Conf. Dr. Cismaş Petrişor (UMF Oradea)
Conf. Dr. Dragomir şerban (UMF Gr. T Popa Iaşi)
Conf. Dr. Grama Maria (UMF Sibiu)
Conf. Dr. Motoc Daniela (UMF Arad)
Conf. Dr. Pumnea Manuela (UMF Sibiu)
Conf. Dr. Suciu şoimiţa (UMF Iuliu Haţieganu Cluj Napoca)
Conf. Dr. Tănasie Gabriela (UMF Victor Babeş Timişoara)
Ş.l. Dr. Bojin Florina (UMF Victor Babeş Timişoara)
Ş.l. Dr. Tatu Carmen (UMF Victor Babeş Timişoara)

You are welcomed with fundamental and applied researches on the following topics:

Inflammation-a double-edged sword
Respiration-from cellular mechanism to systemic process
Stem cells in the biotechnology era
Neurosciences-a multidisciplinary approach
Cardiovascular physiology-from experiment to clinical practice
Physiology curriculum-national harmonization and ECTS integration

The registration and abstract submission will be completed at **http://www.umft.ro/newpage/cnf2012/index.htm** before March 30th, 2012. The Congress is credited by Romanian College of Physicians.