TO THE CHAIRMAN

OF THE SCIENTIFIC JURY,

ASSIGNED IN ACCORDANCE WITH ORDER No P-109-347/ 18.07.2023.

OF THE RECTOR OF THE MEDICAL UNIVERSITY "PROF. D-R PARASKEV STOYANOV - VARNA,

PROF. SVETOSLAV GEORGIEV, D.M., PHD

Dear Mr. Chairman,

Please, find attached: An Opinion Statement on the Dissertation work for the awarding of the scientific and educational degree "Doctor" on the thesis entitled "CYTOGENETIC AND MOLECULAR-CYTOGENETIC MARKERS IN PATIENTS WITH MULTIPLE MYELOMA - PROGNOSTIC SIGNIFICANCE", prepared by Dr. VALENTINA DIMITROVA MITEVA, PhD student in full-time studies in the field of Higher Education - 4. Natural sciences, mathematics, and informatics; Professional field: 4.3. Biological Sciences, Doctoral Program "Genetics" at the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna, Faculty of Medicine, Department of "Medical Genetics", unsubscribed by order of the Rector of the MU - Varna No. R-109-303/16.07.2023.

Reviewer: Prof. Gueogui Nikolaevitch Balatzenko, MD, PhD

Scientific specialty: 03.01.39 - Hematology and Blood Transfusion

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OPINION STATEMENT

by Prof. Gueorgui Nikolaevitch Balatzenko, MD, PhD

Head of the Diagnostic and Consultative Unit, National Specialized Hospital for Active Treatment of Hematological Diseases - EAD, Sofia

Regarding: Dissertation work for the acquisition of the scientific and educational degree "Doctor" on the topic "CYTOGENETIC AND MOLECULAR-CYTOGENETIC MARKERS IN PATIENTS WITH MULTIPLE MYELOMA - PROGNOSTIC SIGNIFICANCE" prepared by Dr. VALENTINA DIMITROVA MITEVA, PhD student in full-time studies in the field of higher education - 4. Natural sciences, mathematics, and informatics Professional direction: 4.3. Biological Sciences, Doctoral Program "Genetics" at the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna, Faculty of Medicine, Department of "Medical Genetics", dismissed by order of the Rector of the MU - Varna No. R-109-303/16.07.2023.

ACTUALITY OF THE PROBLEM

Multiple myeloma (MM) is a complex and heterogeneous hematologic neoplasia characterized by clonal proliferation of plasma cells in the bone marrow. Genetic abnormalities have key role in the pathogenesis and progression of the disease, and this is the reason that the testing for these abnormalities is a mandatory element of the diagnostic algorithm, used for risk stratification, assessment of prognosis and making therapeutic decisions in patients with this disease. Genetic testing in MM involves analysis of various abnormalities, including different chromosomal aberrations, gene mutations, and aberrant gene expression. The results of these studies supply valuable information about the biology of MM in individual patients and help to identify specific subtypes of the disease that may have a different clinical course and response to therapy.

BRIEF DATA ON THE CANDIDATE'S PROFESSIONAL DEVELOPMENT

Dr. VALENTINA MITEVA graduated as a medical doctor at the Medical University (MU) "Prof. Dr. Paraskev Stoyanov" - Varna in 2011 (Diploma No. 001457/2011). Her professional development as a physician began on 01.11.2011 at UMBAL "St. Marina" EAD - city of Varna - Laboratory of medical and molecular genetics, where she worked until 01.03.2012. During the period 15.02.2016 - 15.09.2016, she worked as a resident doctor in the Neurological Department at "MBAL - KAVARNA - OOD" - town of Kavarna. From 03.10.2016 to 31.12.2021, she is a resident physician in the Laboratory of Medical Genetics at UMBAL "St. Marina" EAD – Varna. During the same period, she was also an assistant at the Department of "Medical Genetics at the Medical Genetics University - Varna. After successfully passing an exam, she Bord Certified in Medical Genetics (Diploma No. 025289/01.01.2022) and was appointed as a board-certified geneticist in the Laboratory of Medical Genetics in the same medical institution. Since July 2018, by order No. P-109-429/16.07.2018 of the Rector of MU-Varna, she has been enrolled as a PhD student in full-time training for the development of a thesis for the acquisition of an educational and scientific degree "Doctor" in the field of higher education four. Natural sciences and

informatics, professional field 4.3. Biological sciences and specialty "Genetics". Initially, the topic of the dissertation work was "Predictive genetic biomarkers in certain types of non-Hodgkin's lymphomas" at the Department of Medical Genetics at the Faculty of Medicine of the Medical University of Varna. Subsequently, the topic was changed to "CYTOGENETIC AND MOLECULAR - CYTOGENETIC MARKERS IN PATIENTS WITH MULTIPLE MYELOMA - PROGNOSTIC SIGNIFICANCE"

STRUCTURE AND LAYOUT OF THE DISSERTATION

The dissertation is prepared following the normative requirements and is written on 123 standard pages, illustrated with thirty-nine figures and twelve tables. The dissertation includes the generally accepted sections, distributed as follows: Title page [2 page(s) (#1-2)]; Table of Contents [3 pp. (#3-5)]; Abbreviations Used [4 pp. (#6-9)]; Introduction [2 pp. (#10-11)]; Exposition: I. Literature review [31 pp. (#12-56)]; II. Purpose and Objectives [1 page (#57)]; III. Materials and Methods [16 pp. (#58-73)]; IV. Own research results [14 pp. (#74-87)]; V. Discussion [24 pp. (#88-92)]; VI. Closing remarks [1 page (#93)]; VII. Conclusions [2 pp. (#94-95)]; VIII. Contributions [1 page (#96)]; IX. Appendices [4 p. (#97-100)] X. Scientific publications on the subject [1 p. (#101)]; XI. Bibliography [22 pp. (#102-123)]. The bibliography covers 206 literary sources, of which 5 are in Cyrillic and 201 are in Latin, with 9.2% (n=19) of the cited works published in the last 3 years, and 22.3% (n=46) - in the last 5 years. The autoreferate is written on seventy standard pages and corresponds to the dissertation work.

THE LITERATURE REVIEW includes an analysis of a large number of scientific articles, and presents detailed information on the epidemiology, familial predisposition, and etiological factors in MM. The main staging systems used in the prognostic stratification of patients and the choice of a therapeutic approach are reviewed. Particular attention is paid to the genetic abnormalities in MM, including a wide range of numerical and structural chromosomal aberrations, as well as some characteristic for the disease translocations - t(4;14)(p16.3;q32.33)/-*MMSET/ FGFR3:: IGH*; t(14;16) (q32.33;q23.2)/*IGH::MAF*; t(14;20)(q32.33;q12)/*IGH::MAFB*; t(6;14)(p21.1; q32.33)/ *CCND3::IGH*; t(6;14)(p25.3;q32.33)/*IRF4::IGH* and some others. Besides, particular attention is paid to the abnormalities affecting the first chromosome and del(17p13). In addition, both the classic approaches and markers used in the diagnosis of MM, as well as certain new-generation diagnostic biomarkers, are presented. The AIM of the dissertation is specific "To establish and analyze the type, frequency, and prognostic significance of chromosomal abnormalities in newly diagnosed patients with a clinical diagnosis of multiple myeloma". To achieve this goal, seven TASKS have been formulated: (1) To select the object of the study, and to group it by stages; (2) To establish the frequency and structure of the detected chromosomal aberrations; (3) To analyze the chromosomal aberrations revealed by conventional cytogenetic method and their prognostic significance; (4) To analyze the chromosomal aberrations revealed by the molecular cytogenetic method FISH and their prognostic significance; (5) To analyze the amount of plasma cells in the bone marrow in comparison to the cytogenetic results; (6) To determine survival in relation to cytogenetic results; (7) To perform a comparative analysis of the survival curves depending on the staging system. MATERIALS AND METHODS - The study includes an analysis of 110 adult patients referred for genetic analysis in the Medical Genetics Laboratory of UMBAL "St. Marina" for a period of 5 years (2016-2020). For the dissertation, conventional cytogenetic and molecular cytogenetic analysis (FISH) were applied on cell suspension from bone marrow, with probes against del(17)(p13), t(4;14), t(14;16) and rearrangements in the 14q32 region. For processing, analysis, and documentation of cytogenetic

and molecular-cytogenetic studies applied software products AxioVision SE64 Rel. 4.9.1 and AmScope 3.7. were used. Validated online genetic databases were used in the interpretation of the detected abnormalities. The statistical approaches used are adequate to the aims of the study.

The RESULTS are correctly presented and illustrated with figures, graphs, and tables. The study included 110 newly diagnosed patients with MM (49.1% men/50.9% women), with an average age at diagnosis of 62 years. It has been found that the highest incidence was seen in the age group of 65-69 years (25.45%), and a trend towards an increase in the number of included patients was seen during the study period by about 3.3 times, with an average growth rate of 5.82%. It was reported that based on the International Staging System (ISS) criteria for MM, the largest one was the group of patients with ISS-III stage (48.19%) followed by those with ISS-I stage (25, 45%) and ISS-II stage (26.36%). On the other hand, according to the Revised-ISS (R-ISS), none of the patients were classified as having R-ISS-I stage, while 65.45% were with R-ISS-II stage and 34.55% with R-ISS-III. The analysis of the frequency and structure of the detected chromosomal aberrations shows significant differences in the frequency depending on the ISS stage: a pathological karyotype was found in the I-stages in 3.6%, in the II-stages - in 24.1%, and the III - in 28.3%. Similar correlations were also shown concerning the disorders proven by FISH in the patients grouped according to R-ISS - a pathological finding was seen in 2.8% of patients with R-ISS II compared to 18.4% - in those with R - ISS III. In total, at the time of diagnosis, cytogenetic analysis was performed in 88% of patients with MM, and it was successful in 86% of them. Abnormalities in the number and/or structure of chromosomes were shown in 17% of patients, while a normal karyotype was seen in the remaining cases. In general, among the cases with pathological karyotype, patients with a complex karyotype (64.3%) and those with a single anomaly (35.7%) are distinguished, and the established aberrations are grouped as hyperdiploid karyotype and non-hyperdiploid karyotype. It is reported that in eleven patients the cytogenetic examination was repeated several months after the first testing, however for only one case information concerning the first testing finding and the later changes was provided. FISH evaluated thirty patients, and in 57% of them, a combination of cytogenetic analysis and FISH was applied, while the remaining were analyzed only by FISH. In a total of 24 patients examined for del(17)(p13), and a positive result was found in 9 of them. In none of the patients examined by FISH for a rearrangement in the IGH gene (14q32), t(4;14), and t(14;16), the respective abnormality has been proven. The data from the morphological evaluation of the bone marrow and the relative proportion of plasma cells were compared to the results of cytogenetic studies. Significant differences were found in the findings depending on the degree of myeloma infiltration - in the patients with a normal karyotype, in 46% the infiltration in the bone marrow was <30%, while in all cases with a pathological karyotype, the relative share of plasmatic cells is >30%. The survival rate was analyzed in relation to the cytogenetic results, with an established average survival rate of 41 months. (men) and 28 months. (women). In patients with a normal karyotype survival was 34 months, compared to 8 months in those with chromosomal aberrations. A statistically significant difference was found in the average survival according to the ISS stage - 11 months for patients in the ISS-III stage, 60 months for those in the ISS-II stage and 67 months. - in those in the ISS-I stage, respectively, while no difference was found in the average survival depending on the R-ISS stage - 41 months. in patients in R-ISS-II stage compared to 18 months. in those in R-ISS-III stage. In total, a fatal outcome was found in 52.7% of the patients included in the analysis, reporting a significant difference in mortality according to ISS stages, being the highest in ISS-III stage: 67.92%, compared to 37.93% - in patients with ISS-II stage and 39.29% - in those in ISS-I stage.

In the DISCUSSION, the obtained results are compared with different similar studies. Regarding the obtained results of the demographic indicators, the variations in the average age at diagnosis of MM, which in the present study was 62 years, against the background of the data from the literature, varying between 55 years and 66 years, are noted. The available data about the frequency, structure, and type of chromosomal abnormalities in patients with MM, was compared with the results of the present study. Differences are also highlighted regarding the relative proportion of patients with chromosomal aberrations, which varies between 20% and 66%, compared to 20% reported in the dissertation. The high frequency of complex changes in the karyotype is emphasized and, in concordance with several other, similar studies, it is emphasized that the most often detected abnormalities are trisomies. It is argued that although the demonstration of chromosomal aberrations by conventional cytogenetic analysis is difficult in patients with MM due to the low mitotic index of myeloma cells and variations in the relative proportion of plasmacytes in the bone marrow, the presence of abnormal metaphases is considered as a poor prognostic sign. The advantages of FISH analysis compared to conventional cytogenetics are highlighted, making it a preferred research approach. Data from the literature about the prognostic significance of various chromosomal disorders is also discussed. The prognostic significance of the ISS stage of the disease regarding survival is also emphasized, as the mortality rate increases with the progress of the stage. The most unfavorable is the survival of the patients in the third stage according to ISS. The conclusions in the dissertation are derived from the obtained results and correspond to the tasks set: (1) The comparative analysis of the frequency and structure of chromosomal disorders in newly diagnosed patients with MM, established in the dissertation shows a high correspondence with the data described in the literature; (2) The frequency of detected chromosomal aberrations established by conventional cytogenetic analysis is 17%, and they are divided into hyperdiploid and non-hyperdiploid. The incidence of detected pathology by FISH analysis is 30%; (3) Trisomies predominate in patients with a hyperdiploid karyotype, while structural disorders predominate in those with a non-hyperdiploid karyotype; (4) del(17)(p13) was detected by FISH in 9 patients (30%); (5) A correlation was established between the amount of plasma cells in the bone marrow and the result of the cytogenetic analysis: in all patients with a pathological karyotype, the number of plasma cells was >30%, while in patients with a normal karyotype, the percentage of plasma cells in the bone marrow in 51% was >30%, in 46% it was <30%, and in 3% - a failure to obtain the aspirate occurred ("dry tap"); (6) A statistically significant difference was found regarding the average survival compared to the cytogenetic findings - in patients with a normal karyotype it was 34 months, while in those with a pathological one - 8 months. (p=0.0493). There was no significant difference in median survival between patients with hyperdiploid and non-hyperdiploid karyotype due to the small number of patients. The same association was o regarding the detected chromosomal abnormalities according to ISS stage (p=0.094); (7) A statistically significant difference was observed in survival by ISS stage. Data from the analysis showed a median survival of 11 months for stage III patients. For those in the second stage, it is on average 60 months, and in the first – 67 months. There was no statistically significant difference in survival compared to R-ISS stage (p=0.14). The average survival of patients in the second stages according to R-ISS is 41 months, and in the third - 18 months.

SCIENTIFIC CONTRIBUTIONS

The scientific contributions of Dr. VALENTINA MITEVA are formulated in two main directions:

CONFIRMATIVE CONTRIBUTIONS: (1). The significance of the classification and risk stratification systems as prognostic factors has been confirmed - I accept without remarks. (2). The importance of the application of a conventional cytogenetic method and FISH analysis as routine methods to distinguish low- and high-risk patients with MM has been confirmed - I accept without remarks. (3). The importance of chromosomal abnormalities as a prognostic factor for survival in the Bulgarian population has been confirmed - I accept without remarks.

PRACTICAL CONTRIBUTIONS: (1). For all newly diagnosed patients with MM, it is recommended to conduct a conventional cytogenetic analysis and FISH, which are extremely important in the selection of a therapeutic scheme, as well as for the prognosis - I agree with a remark - the dissertation work lacks own data to argue the importance of the results of conventional cytogenetic analysis and FISH in the choice of a therapeutic protocol. (2). Screening for t(4;14) and del(17p), which have an unfavorable prognosis in newly diagnosed MM patients, is recommended as a mandatory element of the diagnostic panel tests - I agree with a remark concerning the section on the unfavorable prognosis of t(4;14) – there is no objective own data to demonstrate the unfavorable prognosis of the anomaly, thus to be an argument for this statement. (3). It is recommended that the analysis for t(4;14) and del(17p13) should be performed by FISH - I agree with a remark concerning the section that recommends the examination of t(4;14) by FISH - The conclusion is entirely speculative, based on literature review, as there is not a single patient carrying t(4;14) identified by FISH in the dissertation. In addition, although the FISH methodology is the most widely used practical approach for the diagnosis of the anomaly, alternative approaches can also be used for this purpose: and polymerase chain reaction (PCR) [Li F, et al. Oncotarget.2017;8(31):51608-51620.] and next-generation sequencing [Rosa-Rosa JM, et al. Cancers (Basel). 2022;14(20):5169]

ARTICLES RELATED TO THE DISSERTATION

The attached list of scientific works related to the dissertation work of Dr. VALENTINA MITEVA includes five full-text articles, of which three are original articles, incl. 2 in foreign scientific journals and 1 – in a Bulgarian magazine, and two literary reviews published in refereed Bulgarian journals. On three of the papers Dr. Valentina Miteva is the first author, on 1 - second, and on 1 - fourth: Original articles: (1) Miteva V, Ruseva C, Chervenkov T, Micheva I. Cytogenetic findings in 126 patients with Multiple Myeloma – a retrospective single-center study. Archives of Hellenic medicine 2023:40(3):349–355. The journal is indexed in the international database SCOPUS. [impact factor (IF) - 0.111 (2022/2023) [60 points (pt.)/4=15)]; (2) Yahya D, Miteva V, Micheva I, Ruseva Ts, Angelova L. Cytogenetic analysis of patients with hematological malignancies. Cytology and Genetics, 2023:57(3):272-281. The journal is indexed in the international database SCOPUS. [IF 0.5 (2022)] [60 pts./5=12)]; (3) Miteva V, Ruseva Ts, Chervenkov T, Micheva I. Chromosomal abnormalities, and their predictive significance in patients with multiple myeloma. Medical Review 2022:58(1):38-43. The journal is indexed in the international database Web of Science (CABI) [60 items/4=15)] Literature reviews on the issues of the dissertation work: (4) Miteva V, Khachmerian M, Chervenkov T, Micheva I. Cytogenetic aberrations in patients with multiple myeloma. Medical Review, 2021:57(5):28-35. The journal is indexed in the international database Web of Science (CABI) [60 items/4=15)]; (5) Khachmerian M, Levkova M, Stoyanova M, Miteva V, Angelova L. Challenges of medical genetic counseling in the era of genomic medicine. General Medicine 2019:21(6):37-40. The journal is indexed in the international databases Scopus and Web of Science (CABI) [60 items/5=12)] Total number of points: 69, with a required minimum number of 30 [sum of Group

D indicators from 5 to 9] according to the Minimum National Requirements for the Scientific and Teaching Activity of Candidates for the Acquisition of the Doctoral Educational and Scientific Degree. The scientific activity in relation to the thesis also included two scientific reports on the topic of the dissertation presented at prestigious international scientific forums: (1) Lukanov R, Dimitrova S, Miteva V, Micheva I. Conventional cytogenetic analysis in patients with multiple myeloma-single center study. Abstract Book HemaSphere, 2021:5: S2: p.797; (2) Miteva V, Ruseva T, Hachmeriyan M, Levkova M, Angelova L; A retrospective study of patients with Multiple Myeloma tested in the Laboratory of Medical Genetics, Varna, Bulgaria; European Journal of Human Genetics, 2020:28(SUPPL 1):940-941.

CRITICAL NOTES

• The literature review is thorough and contains diverse and detailed information on various aspects of multiple myeloma. At the same time, a significant part of this information is not related to the implemented experimental set-up and the conducted research. On the other hand, there is a lack of a clearly defined opinion about the unsolved problems in the respective area of research, as a basis for the realization of the research activity and no formulated scientific hypothesis.

• There are no data on the presence, type, and amount of paraprotein and free light chains in the serum and urine of individual patients; the presence and severity of organ involvement – bone disease, renal dysfunction, the presence, and severity of anemia, which are relevant to the clinical course of the disease.

• In general, the number of patients with proven chromosomal abnormalities by conventional cytogenetic analysis is too small – only fourteen. Considering the heterogeneity of the aberrations found, only the prognostic significance of the presence of pathological karyotype could be analyzed, but not of the individual anomalies.

• The situation is similar in FISH studies - a total of thirty patients, with relevant results being obtained only for del(17)(p13) - a total of 21 patients were examined, with a positive result found in 9. The patients examined for the other anomalies are extremely insufficient for any conclusions - a rearrangement in the IGH gene in the 14q32 region was carried out in 6 patients, the translocations t(4;14) and t(14;16) – in 15, without a pathological finding, the result was normal in all, which is not unexpected, but given their prevalence, respectively 10-15% and 6-7%.

• Repeat cytogenetic testing was reported in eleven patients; however, the respective findings were reported in only one case. In the remaining ten cases, no information was presented concerning the first cytogenetic status, as well as the subsequent findings.

• The dissertation discusses the survival of the patients with MM; however, no data was given on whether they were treated or not, and what therapeutic protocols were applied. All this does not allow us to figure out the prognostic significance of the respective abnormalities.

• The designation of gene fusions formed by translocations is not in accordance with the recommendations of the Human Genome Organization (HUGO) Gene Nomenclature Committee (2021), according to which all fusions, whether in the form of hybrid, chimeric genes or those in which regulatory genes of one gene lead to deregulation of the other partner gene should be indicated by (::) [Bruford EA, et al. Leukemia 35.11 (2021): 3040-3043].

CONCLUSION

Based on the above, I believe that regardless of the critical remarks, the dissertation presented by Dr. Valentina Dimitrova Miteva meets the specific requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria and the "Regulations on the Development of the academic staff at the Medical University - Varna, which gives me the ground to give a positive opinion of the dissertation work and to propose to the honorable Scientific Jury to award the educational and scientific degree "DOCTOR" to Dr. Valentina Dimitrova Miteva.

14 September 2023

Prof. Gueorgui Balatzenko, MD, PhD