To the Chairman of the Scientific Jury,
Appointed Order № P- 109- 377/06. 10. 2020 of
The Rector of the Medical University -Varna

REVIEW
FROM
PROF. DR. LYUDMILA BONCHEVA ANGELOVA, MD, PhD, DSc
HEAD OF THE DEPARTMENT OF MEDICAL GENETICS, MU-VARNA

ABOUT:

Dissertation on the topic **" Clinical application of prognostic factors and their integration into a scale for risk assessment and time to treatment in untreated patients with B-chronic lymphocytic leukemia"**
of **Dr. Vania Slavcheva Popova ,** a PhD student in independent form of education in the Sector of Hematology, Second Department of INTERNAL diseases MU Varna , enrolled in a doctoral program in "Hematology and blood transfusion" 7. Healthcare and sports, professional field 7.1 Medicine for awarding the educational and scientific degree **"PhD"**

Scientific Supervisor : Prof. Dr. Liana Todorova Gercheva - Kyuchukova , MD, PhD, DSc

The doctoral student was enrolled by Order № P-109-126 / 01.04.2019 of the Rector of the Medical University "Prof. Dr. Parsakev Stoyanov" Varna. The review was prepared in accordance with the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for the Implementation of the Law on the Development of Academic Staff and the Regulations for the Development of the Academic Staff of the Medical University of Varna . The fulfilment of the doctoral program have been observed, there is no change in the initial topic and the supervisor.

Brief biographical data about the doctoral student:
Dr. Vania Slavcheva was born and over 1965 in Pleven, graduated Medical University – Pleven in 1990. She acquired a specialty in Internal Medicine in 1997 and Clinical Hematology in 1999. For 24 years (1996 - present) she has been working as a physician - assistant (1996 - 2000), senior (until 2014) and assistant (2015 until now) at the Clinic of Hematology at the University Hospital "Dr. G. Stranski" Pleven.
She graduated in internal medicine in 1997 and Clinical Hematology in 1999. For 24 years (1996 - present) she worked as a doctor - assistant (1996 - 2000), senior (up to 2014) and assistant (2015 to now) in the Clinic of Hematology of the University Hospital "Dr. G. Stranski" Pleven. She acquired first professional skills and competencies appropriate to implement the teaching of hematology to medical students, both Bulgarian and English-language training; co-author of 22 full-text publications in Bulgarian and foreign journals with 60 citations. There is no information about specializations and courses abroad.

The doctoral student participates in 2 research projects on the problems of B-chronic lymphocytic leukemia, funded by the Science Fund MU Pleven. This fact, the prospective approach of the study in the period 2016-2019, as well as 4 publications from the last three years show an increased scientific interest and focus on molecular genetic markers, after publication on the prognostic value of an immunological marker (2005) of this type of leukemia.

Structure of the dissertation

The dissertation presented is structured according to the accepted requirements. It contains a total of 123 standard pages, illustrated with 49 figures and 30 tables and 1 appendix, distributed as follows: Title page (1), Contents (1), Abbreviations used (2), Exposition: a) Literary review (33), b) Working hypothesis together with purpose and tasks (1 -2) c) Material and methods (9), d) Results (30), e) Discussion (18) f) Conclusion (1), g) Conclusions (1), Contributions (1) Appendices (5), Publications and participations on the topic (1) Bibliography (19). The bibliography includes 199 literary sources, of which 5 in Cyrillic and 194 in Latin; of all of them at least one - the third are from the last 5 years. The abstract is written on 64 standard pages in accordance with the dissertation.

Relevance of the topic of the dissertation

The dissertation submitted for review addresses a topical issue due to the need for improved risk stratification and modernization of the approach for treatment of chronic lymphocytic leukemia. The original idea of studying patients for the so-called clinically significant cytogenetic aberrations that define resistance to immunochemotherapy are insufficient to stratify the risk of progression and treatment choice, as they are extremely variable over the course of the disease. For this reason, the research team suggests the participation of another prognostic and predictive marker, IGVH mutation status, which is determined by the method of new generation sequencing. For its evaluation, it is proposed to use an alternative approach through the participation of substitute markers (by the author "surrogates") to determine this status by simultaneous detection of the products of ADAM29 and LPL genes by multiplex PCR analysis. The proposed consideration of the molecular genetic profile (determining the course of the disease) aims to adapt to the proposed International Prognostic Index for a more complex assessment of the time to treatment in untreated patients with chronic lymphocytic leukemia.
Given the above, I believe that the study of prognostic biomarkers and their integration into a scale for risk and time to treatment in untreated patients with B-chronic lymphocytic leukemia is relevant, mainly of scientific importance.

1. Literature review The dissertation begins with an overview of B-cell development and biology of B-CLL - definition, clinical course, prognostic factors. This information should be presented as an Introduction and Literary Review on the topic, what is its essence. In this

section the author systematizes the scientific data and shows good information in two subsections 1) Historical and current review of the biology of the disease B-chronic lymphocytic leukemia and 2) Clinical course, classical and new prognostic factors. The section is in-depth and propaedeutically illustrated with 12 figures and 10 tables.

Chronologically studied from 2008 to 2018, the recommended genetic and molecular markers from being just recommended indexes tend to be established as leading with the idea of classifying CLL based on the mutational status. Conventional cytogenetic analysis of the peripheral blood lymphocytes (with stimulation)general is not mandatory, but according to 2018 reccomendations it is proposed that for the purposes of clinical practice in CLL to always apply the following major genetic disorders with known prognostic value a) molecular cytogenetics by FISH for del(13q), del(11q), del (17p), add (12) of peripheral blood lymphocytes, b) TP53 mutation and c) IGVH mutation status (Hallek M, Cheson BD, Catovsky D).
Despite the presence of more than 35 prognostic factors, for most of them there are no generally accepted and established standard methods for research.
After summarizing the review, Dr. Slavcheva logically derives the working hypothesis, for which she gives valid arguments for the choice of the researched topic - the complex study of known phenotypic and the inclusion of more modern genetic (chromosomal and molecular deviations) prognostic factors with clinically proven value to more accurately stratify risk and predict the time to treatment need in patients with newly diagnosed B-CLL.

Based on the presented data and the analysis of the condition of the prognostic markers, the postgraduate clearly shows the need to conduct research in our country related to complex assessment of, correctly specified, available to our country molecular genetic in addition to classical prognostic factors. To this end, a new prognostic marker of lipoprotein lipase A expression and ADAM29 expression was added to established molecular cytogenetic disorders (requiring future studies, along with HS1 protein expression, mir RNA, CLLU1) to replace the extensively studied IgVH mutation status 2006.).
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2.The goal *is well-formulated and clear - to explore and analyze the impact of available clinical, laboratory, molecular and genetic factors related to the specific characteristics of both the patient and his disease, and to assess their impact on the time for initiation of treatment in untreated B-CLL patients.*
To achieve this goal, *6 tasks* are formulated which are logically connected and presented in a synthesized form. A matter of professional interest are fourth, fifth and extent sixth tasks, which attract mainly my attention:
1. To investigate the demographic factor of age and its influence on the time to initiating treatment in patients with untreated B- CLL
2. To analyze the significance of the stage of the disease as a prognostic factor and its association with TTFT.
3. To search for a correlation between the serum marker β-2 MG and the time to start treatment.
4. To investigate the frequency of some clinically significant chromosomal aberrations in

untreated patients with B-CLL and their distribution, based on the stage of the disease.
5. To determine the mutational status using the surrogate markers LPL, ADAM29 and identify a possible correlation between the molecular and genetic changes in patients with untreated B-CLL.
6. To make a comprehensive assessment of the prognostic factors and their importance in determining the time to start treatment

3. Material and methods

The section starts as a prospective and retrospective study within three years 2016-2019, and includes a clinical contingent studied in the units of two university hospitals: A) University Hospital "Dr. Georgi Stranski" - Pleven with several units (Hematology Office, Clinic of Hematology, Central Clinical Laboratory, Medical Laboratory of Immunodiagnostics, Clinic of Imaging) and B) Laboratory of Molecular Biology and Cytogenetics at NSHATHD - Sofia. The study included patients with documented B-CLL over the age of 18 in three groups.

Group 1 included 97 patients in whom standard laboratory tests, flow cytometric analysis of peripheral mononuclear cells, beta 2-microglobulin level (studied in 93 patients) and staging procedures were performed at diagnosis of the disease.

Group 2 included 61 randomly selected from patients from group 1. On these patients, fluorescent *in situ* hybridization was performed.

Group 3 included 48 patients selected at random from group 2. These patients underwent additional PCR analysis

The research methods used by the doctoral student for clinical phenotyping could be devided as: a) Documentary method b) Flow cytometric screening for selection of the target patient group, c) Instrumental (imaging diagnostics) d) Laboratory (clinical-laboratory, immuno diagnostic)

The multi-disciplinary nature of scientific research is evident from the significant importance of genetic laboratory research through molecular cytogenetics (FISH) and through molecular genetics - multiplex polymerase chain reaction after reverse transcription of RNA into complementary DNA. All methods, incl. laboratory protocols are described in details, with the necessary sequence, corresponding to the stages of the study and according to the requirements of the dissertation.

Remarks.

The groups indicating the distribution by type of study are a basic characteristic of the patients and should be in the section "Clinical contingent and methods". The correct number of examined patients from group II (by fluorescent in situ hybridization) is 62, and they include 1 with non-presentative fluorescent signals (in itself a very good methodological success).

4.The results are described in a logical maner and are illustrated in detail to correspond to the set tasks with tables and figures. According to the type of questions asked for resolution and the conducted studies, the results are grouped in 3 sections. A detailed characterization of both the demographic and clinical-laboratory features, as well as genetic ones is presented:

- **Demographics factors** and their influence to TTFT
- **Clinical and laboratory indicators** determining the stage of the disease and related to TTFT
- **Molecular genetic markers** (fluorescent in situ hybridization and multiplex polymerase chain reaction after reverse transcription)

The analysis did not reveal a significant difference in time for begining the treatment between the two age groups 57-67 years. and 68-78. (Kaplan - Meier, p = 0, 596) (Fig. №.17) and did not show a statistically significant difference (p > 0.05) between the two sexes and TTFT (Fig. №21) . In summary, the influence of demographic characteristics age and sex on the time from diagnosis of the disease to its initial treatment is not established. The staging of the patients was performed on the basis of a complex assessment of the results of the physical status, imaging examinations (ultrasound of abdominal organs, computed tomography, X-ray of the lungs) and routine laboratory tests . The statistically significant difference in time to treatment between groups of patients (log-rank test, p <0.001, Fig. №23), the shortest for patients in stage C (advanced) and the largest in patients with stage A - confirm the role of the clinical stage as a significant factor associated with TTFT .

A relationship between the serum marker β2 - MG as an indicator indirectly reflecting proliferative activity and tumor volume, and to what extent the factor can be defined as significant for TTFT (a single prognostic factor related to time to first treatment) was sought. There was a tendency for a weak negative relationship between the determined concentration of beta 2-microglobulin and the time from diagnosis to treatment for patients in stage A (Rs = -0.213, p = 0.086), ie. the higher concentration of the marker is associated with a shorter time till treatment initiation(diagnosis - initiation of treatment) (Fig. №31) .

Genetic testing for prognostically significant chromosomal aberrations of three selected markers was performed in 62 patients over a three-year period. For obvious reasons, interphase FISH analysis and once carried out , and not on all 97 patients, but is appropriately applied as a kind of tags and results adequately analyzed in 5 subgroups (isolated del13q ; isolated del11q; del17p / p53 ; more than one chromosomal aberration ; none of the above-mentioned markers is found). The most frequent chromosomal abnormality is del13q (at 42.6%), significantly less frequently del17p / p53 (9,8%), and 22 (36.1%) of the patients do not have chromosomal abnormalities. Here it should be reported and patients with isolated del11q. The figures related to the FISH signals for del17p for the p53 gene (incl. monosomy 17), del11q for the ATM gene, del13q14) / 13qter for the DLEU1 gene are very clear and demonstrative . I find Table № 23 and Fig. 39 very suitable for presenting the results in this subsection of results.

The PhD student analyzes the most common chromosome marker - isolated del13q (pg.70), searching for a relationship between the percentage of aberrant cells and the time to start treatment. There was no found statistically significant difference between the time to treatment of the patients from the conditional two groups (p> 0.05) in this group of 26 patients.

The molecular genetic results of the multiplex PCR analysis after reverse transcriptase on RNA for lipoprotein lipase A expression and ADAM29 expression as alternatives for assessing IgVH mutation status are of good interest. The illustration from the performed analysis (Fig. № 42) is demonstrative, although presented as a " sample of the results of the performed PCR analysis" . Attention is drawn to the results of the research for the relationship between mutation status and chromosomal abnormalities (Table № 25) . It turns out that not only is the number of patients with del13q is the highest, but also that this aberration is the

most represented among patients with mutated IGVH status. The analysis data display the presence of significant correlation between the mutational status and test karyotypic aberrations (p = 0.035). The chromosome marker del17 p, the presence of which is associated with an unfavorable prognosis, was observed in 12.5% (2/16) in the group with unmutated IGVH status, *without specifying the statistical significance of the relationship.*
The distribution of patients according to the mutational status and time to initiation of treatment TTFT (Table.№ 26 and № Figure 43) is to display the finding by comparing the curves Kaplan - Meier .

I accept the indicated results and their statistical processing as adequate to the goal and the tascs of the research.

Remarks
I think it`s logically , that the result for Distribution patients at risk related to the category of TTFT (based on scales of risk assessment, proposed in CLL - IPI) and "transcode prognostic factors", to be separated in the Subsection of the Results chapter.
5.In the **Discussion section**, the doctoral student analyzes the results obtained, showing in-depth knowledge and searching for logic by integrating prognostic factors into a scale for risk assessment and time to treatment. It goes beyond hematological knowledge and discusses interdisciplinary links with cytogenetic and molecular genetic studies not used in mass clinical practice. All results are commented in pathogenetic aspect on the basis of modern knowledge and similar studies in the medical literature.

An association between cytogenetic disorders and mutational status has been sought . The PhD student upgrades the chromosomal markers and skillfully focuses on IGHV mutation status as a factor that does not change in the course of the disease and has the value of a prognostic and predictive marker for more accurate assessment of the risk of progression among untreated patients *(Moia R et al, 2020*). Although in a small group of patients, the authors attempted to validate CLL - IPI using a surrogate mutation status marker. Its substitute in the present work is a study of lipoprotein lipase A and ADAM29 expression, which were proposed by Emili Montserrat, 2006 in the category " requiring future studies " together with the expression of HS1 protein, mir RNA, CLLU1 expression.

In the clinical studies, including large patient groups, an increased incidence of chromosomal aberrations associated with an unfavorable prognosis (11q, 17q-) among those with unmutated IGHV status (*Zenz T, Dohner H 2011)* is shown*; in* 40-50% of untreated patients, unfavorable prognostic factors (del17p, del11q , unmutated IGHV status) are found *(Parikh S 2018).* In the present study, there is a link between an increased frequency of cytogenetic markers and mutational status , but the doctoral student realistically finds that the number of groups of patients studied is a limiting factor to be able to draw definite conclusions. An important factor is the heterogeneity in the study phase (between diagnosis and treatment initiation), the limited number of patients in the high-risk group, the unbalanced distribution of patients by disease stages for the individual subgroups; time to determine the laboratory

parameters (beta-2-microglobulin, ABLC) relative to the time of diagnosis and determination of the stage .

Of particular interest is the cited state-of-the-art multicenter, prospective study of the German CLL study group on patients in early A clinical stage, which confirms the unfavorable prognostic value of the factors: age over 60 years, LDT under 12 months, elevated β2-MG values. , del11q, del17p, unmortal IGHV status, and the inclusion of both factors del11q and LDT leads to a proposal for CLL1-PM as an opportunity to assess TTFT and OS in patients with untreated early-stage CLL (Hoechstetter MA et al, 2020).

In conclusion, it is correctly noted that the addition of some of the many molecular and genetic markers that reflect complex processes in neoplastic cells provides an opportunity for both better assessment of the risk of progression and as prognostic factors in patients with untreated B-CLL. It is strongly recommended to be used in clinical practice for assesing the time for initiation of treatment and the risk of progression.

6. **Conclusions and contributions.** The conclusions follow the tasks set in the dissertation.

1. The median age of the newly diagnosed patients with B-CLL in our study was 67 years. The age is not a factor that influenced the time to first treatment.
2. The patients with early stages of the disease were the largest part of patients with B-CLL, who visited the Hematologic Outpatient Consulting Room and the Clinic of Hematology.
3. The clinical stage of the disease was a significant factor associated with TTFT.
4. High serum levels of β-2 MG correlated with shorter time to treatment. In patients at the early stage, a negative correlation between the values of the indicator and TTFT was established.
5. The absolute B-cell lymphocyte count above 50G/l at the diagnosis is associated with the possibility of early progression.
6. The presence of del11q and del17p, or a combination of more than one chromosome aberrations, including in combination with del13q, was associated with a shorter TTFT.
7. The PCR analysis allows to identify high-risk patients with a UM status, in which the mean time to initiation of treatment is significantly shorter than TTFT in subjects with MT status
8. Del13q was observed more frequently in patients with mutated status and was associated with a favorable prognosis, unlike the structural aberrations 11q – and 17p – , which were detected in subjects with unmutated status and correlated with short-term TTFT. The prognostic value of 13q- was different in cases where it was combined with unmutated status.
9. In more than one-third of the patients with an early stage of disease adverse prognostic factors were observed: del17p and UM status. This finding proves the need to determine them at the time the diagnosis is made.
10. The complex assessment, based on the molecular-genetic markers available for research in patients with untreated B-CLL provides a better opportunity to stratify the risk of progression, determine the time to treatment, the period for follow- up and, accordingly, the choice of the most adequate therapy.

The author presents and two applications related to practical approach for testing and informing patients surveyed Annex 1 (informed consent) and Appendix 2 (Questionnaire) .

Remarks. I find that some of the conclusions need refinement (6,8,9), clarification (3) or unification in order not to sound like a statement (1,2).

The contributions are represented by PhD in 3 groups.

Contributions of original character

- ✓ For the first time, in our country the relationship between unfavorable molecular genetic factors in patients with untreated B-CLL and their influence on the time to initiation of treatment was studied , which in turn allows the choice of the most adequate modern therapy.
- ✓ Stratification of patients with untreated B-CLL was performed based on the integration of a surrogate marker to classical prognostic factors and chromosomal aberrations.

Contributions of a confirmatory nature

- ✓ The importance of the stage of the disease and its determining influence on the time to treatment has been confirmed.
- ✓ The presence of a correlation between the levels of the serum beta-2 microglobulin and the time to treatment was confirmed.
- ✓ A need for assessment of molecular and genetic factors associated with the risk of disease progression and risk-adapted therapy, respectively, has been confirmed.

Contributions of an applied nature

- ✓ A questionnaire containing information about the patient and the disease has been prepared.
- ✓ An approach has been developed for screening high-risk patients with untreated B-CLL .

Accept contributions formulated by the following January Comment

In my opinion, the most significant contribution of the dissertation is the clinical phenotyping by the author and his attempt to interpret the observed phenotype in the context of the results of molecular genetic research *in a scientifically applied aspect* . And the use of RNA- based surrogate markers, as an alternative to determining mutational status, can serve to more accurately assess the risk of early disease progression only at this stage , before the accelerated introduction of molecular analysis by new generation sequencing for the purposes of clinical practice.

As a geneticist, I can decide whether it is sufficiently acceptable use of surrogate us of mutation status and chromosome aberrant our marker and to validate not CLL - IPI and score more in the diagnosis of disease (in arguably valuable information for clinics cyst) in a small group of patients . This leads me to suggest that the applied contribution "An approach to screening high-risk patients with untreated B-CLL has been developed" sounds like a variant of a "rational approach to detecting high-risk patients with untreated B-CLL ".

7. Papers related to the dissertation a

The enclosed list of scientific papers related to the thesis includes four full-text articles , all co-authored, and two messages in scientific forums make a good impression that the PhD student is lead author in av them all, and that the publication in J ournal of IMAB (Scientific papers, Annual Proceedings) is cited in a reference journal with an impact factor.

8. Critical remarks and recommendations

In essence , I have attached notes and comments to the relevant section . Here I could mention some technical and stylistic recommendations for consideration in future research papers of the doctoral student.
Regarding the structure - no Introduction; The list of *abbreviations* used *is* too large and untidy; the sections " Research Materials and Methods " (shown but above logic) " Research Results " " Discussion " can be presented more clearly in Contents and in Text. The so-called Place and time of the study is actually the beginning of a generally accepted clinical contingent.
It is better to avoid duplication of information (Fig. 26 and Table 19, Fig. 19 and Table 16), inappropriate scientific writing of molecular cytogenetic markers (17p-, 11q-, 13q- (p. 48,70,77) incomplete description of the FISH signals (Fig. №38, №36, №40) according to the accepted nomenclature for the cited patient.
Inaccuracies in the spelling of the Literary Sources, the lack of uniformity and completeness I attribute to the lack of technical time of the doctoral student: (Savov, A., Petrov B, Kirova et al. In: Predictive biomarkers in oncology. Volume 1, Solid tumors instead of correct authors Balatsenko, GM Genova in: Predictive biomarkers in oncology, Volume 2, Hematological neoplasms from the same year 2017).

8. Conclusion:

The dissertation demonstrates the doctoral student's knowledge not only in the field of her clinical specialty, but also in the field of molecular biological sciences and their application to medical practice. The research is up-to-date and meets the scientometric criteria in accordance with the regulations for academic development of MU-Varna for awarding the scientific-educational degree "Doctor". I recommend the scientific jury to award the scientific-educational degree "Doctor" in the scientific specialty "Hematology and Blood Transfusion" to Dr. Vanya Slavcheva Popova.
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28.10. 20 20 Member of NJ - Reviewer:

PROF. DR. LYUDMILA ANGELOVA, MD, PhD, DSc