

## Transcriptional activity of telomerase complex in CD34<sup>-</sup> stem cells of cord blood in dependence of preparation time

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**Abstract:** The aim of the study was to determine whether the expression of telomerase subunits encoding genes changes during the process of cord blood preparation. It should establish if the commonly accepted 24 hours time interval in stem cells kriopreservation procedure significantly influences their immortalization and so decreases the "quality" of cord blood stem cells. Investigation includes 69 women. Spontaneous labour was the inclusion condition. The material was collected at birth after clamping of umbilical cord by direct vasopuncture. CD34<sup>-</sup> cells were extracted from cord blood (MACS, Miltenyi Biotec; Bisley, Surrey, UK). The expression profile of telomerase activators and inhibitors encoding genes was determined using HG\_U133A oligonucleotide microarray (Affymetrix). We used a real-time quantitative RT-PCR assay to quantify the telomerase TERT, hTR and TP1 subunits mRNA copy numbers in CD34<sup>-</sup> cells in 0, 6, 12 and 24 hours after cord blood collection. We observed significant decrease of numbers of copies of TERT+B mRNA within the successive hours of observation. Significant decrease of numbers of TERT A mRNA copies was confirmed after 24 hours. However, we observed significant increase of numbers of copies of TERT B mRNA after 6 hours of observation. Similar level was maintained during another 6h. The significantly lower number of copies of TERT B mRNA was observed after 24h. We also observed significant increase of number of copies of TERT mRNA after 6 hours. Number of copies of TERT mRNA significantly decreased after another 6h, remaining, however, on a higher than initial one. The significant lower number of copies of TERT mRNA was observed 24h after delivery. The possible explanation of those results is discussed in the paper.

**Key words:** cord blood, stem cells, microarrays, telomerase subunits encoding genes, transcriptional activity.

### Introduction

The fast developing life science and genetics in the last century brought to appearance of new therapeutic methods leaning towards the use of cord stem cells. Research over improvement of cell cultures methods, composition of mediums, and use of various factors stimulating cell proliferation (growth factors, cytokines, chemokines and hormones) are bringing

medicine closer to the wide clinical use of stem cells. Immortalization, which means preservation of telomeres after each and every stem cell's division, is one of characteristic features of stem cells determining their potential use in medicine. The factor that prevents telomeres shortening after division of a stem cell is telomerase. Also normal somatic cells such as hemopoietic cells, cervical epithelial cell and endometrial cell, all of which have high regenerative potential, are also known to express telomerase activity.

Telomerase is a reverse transcriptase which adds repetitive telomere sequences to the end of chromosomes which is thought to be essential for cellular immortality. The enzyme consists of three subunits:

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hTERT (*human telomerase reverse transcriptase*) (synonym TCS1, EST2) - a subunit of an activity of reverse transcriptase; hTR (*human telomerase RNA*) (synonym TERC, TRC3, TR) of the matrix section of RNA; and TP1 - an accompanying protein of an unknown function. The hTERT subunit determines the activity of telomerase as an enzyme. Many studies have revealed that hTR and TP1 is expressed constitutively. The presence of TERT and hTR subunits makes possible the activity of telomerase *in vitro*. This second subunit is the matrix for the hTERT polymerase. hTR is an RNA fragment complementary to telomere sequence. In the cell is postulated the presence of whole complex which controls telomerase activity. Among the proteins binding with hTERT and hTR highlighted the TP1 protein (*telomerase protein 1*). TP1 has ability to bind to hTERT and hTR.

Telomerase activity is connected with cells proliferation and differentiation. The majority of regular human cells do not show an expression of a gene of hTERT subunit or the enzymatic activity of the complex, despite the presence of the other components. Only the expression of a gene for the catalytic subunit hTERT enables cells to demonstrate the telomerase activity. This observation proves that the hTERT subunit is a determining factor of the *in vivo* activity of the entire complex. The control of hTERT expression is crucial to extension of proliferation and immortalization potential. Therefore, it is accepted that one of the markers of immortalization activity of cord blood stem cells is the presence of the telomerase complex, especially its hTERT subunit together with its functionally vital isoforms A and B.

In this study we presented the transcriptive activity of telomerase complex of cord blood CD34<sup>-</sup> stem cells with respect to the duration time of preparation process in healthy pregnant women. The important problem concerning stem cells is the preparation process which takes place between their collection and freezing. Within the over a dozen years of existence of cord blood banks all over the world, it was agreed that the entire preparation process should be finished within 24h from collection. This is due to the hypothetical decrease of stem cells vitality with time after the collection. Duration of that procedure and its influence on the "quality" of frozen material is the subject of this study.

The aim of the study was to estimate the dynamics of change in stem cells immortalization potential during preparation of cord blood by measuring the expression of telomerase subunit encoding genes in cord blood stem cells directly after collection as well as 6, 12 and 24 hours after collection. It should allow establishing if commonly accepted 24 time interval in procedure of stem cells kriopreservation is suitable for their "quality".

## Materials and methods

**Patients.** Research group included 69 healthy patients between 18 and 35 years old, hospitalized in the Department of Obstetrics and Gynecology, Medical University of Silesia, Katowice, Poland. The inclusion criteria were spontaneous physiological labor and no health problems before or during pregnancy. Among that group, 35 women agree to participate in the research. Out of those 35 patients, 22 women were primiparas, 12 patients labored for the second time and 1 woman for the third time.

**Cord blood preparation.** The studied material was taken at birth and after clamping of umbilical cord by direct vasopuncture of umbilical vessels after previous sterilization of a place of puncture. The entire procedure was held before the end of third stage of labour. Human umbilical cord blood samples were collected directly into sterile tubes with reduced content of anticoagulant. The blood was transported to The Department of Molecular Biology in accordance with standard procedure executed through the cryopreserve centres.

Studied material was quartered. First part was frozen in -20°C just after collection, second six hours after, third twelfth hours and fourth twenty four hours after collection.

**CD34<sup>-</sup> cells extraction from cord blood.** The cord blood was diluted with PBS (Gibco) and in proportion 2:1 with Ficoll (Sigma). Diluted cord blood was centrifuged at 400xg for 40 minutes in 20°C. A Peripheral Blood Mononuclear Cells were then transferred to a medium with addition of serum and centrifuged at 200xg for 10 minutes in 20°C.

The cell suspension was incubated with blocking reagent and a CD34 antibody from the CD34 Isolation Kit (MACS, Miltenyi Biotec; Bisley, Surrey, UK) for 15 min at 4-8°C. After incubation, the cells were washed in PBS and resuspended in 400 µl of the same buffer. 100 µl of immunomagnetic bead suspension conjugated to an antimouse antibody were then added to the cells, and incubation continued for a further 15 min in the cold. After washing, the CD34<sup>+</sup> cells were isolated by passing the cells suspension through a column placed in a magnetic field which allowed retention of the target cells. The magnetic field was then removed, and the CD34<sup>+</sup> cells were flushed from the column with PBE buffer. The number of cells was then determined by fluorescence measurement after incubation of CD34<sup>+</sup> cells with anti-CD34 (BD) monoclonal antibodies conjugated with IgG1 antibodies (Becton Dickinson). Amount of leukocytes, mononuclear cells, erythrocytes and trombocytes was determined by haematologic analyser Sysmex (Toa Medical Electronic). Cells vitality was marked in fluorescent microscope after labelling by ethidium bromide and acridine orange. Number of CD34<sup>+</sup> cells was analysed by flow cytometry using monoclonal antibody anti-CD 34 coupled to phycoerythrin or IgG1 antibody (Becton Dickinson). Fluorescence was then analyzed by FACSCalibur™ flow cytometer (Becton Dickinson) and CELLQuest™ software (Becton Dickinson). The analysis was carried out with the use of FACSCalibur (BD) device and CELLQuest (BD) program. CD34<sup>-</sup> cells were isolated in Department of Experimental Hematology of Oncology Institute, Warsaw.

**CD34<sup>-</sup> cells culture.** The CD34<sup>-</sup> cell line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco Invitrogen) with penicillin/streptomycin and 5% bovine serum albumin.

**RNA extraction.** Total cellular RNA was extracted from CD34<sup>-</sup> cells using TRIzol (GibcoBRL). All extracts were treated with DNA-se I to avoid contamination of genomic DNA. RNA was purified on RNeasy Mini Kit columns (Qiagen). RNA extracts were qualitatively evaluated by electrophoresis in 1,5% agarose gel and quantitatively by spectrophotometry (Gene Quant II by LKB Pharmacia Biotech).

**Microarray Assay.** The oligonucleotide microarray method is the semi-quantitative analysis. The oligonucleotide microarray technology platform simultaneously analyzes the relative expression levels of genes. The expression profile of telomerase activators and inhibitors encoding genes was determined using HG\_U133A oligonucleotide microarray (Affymetrix). In order to obtain the first thread of cDNA, to 8 µg of RNA 1 µl 100 pM of starter T7-oligo (dT)<sub>24</sub> (5'-GCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-3') was added, the mixture was incubated at 70°C for 10 minutes and placed in ice. Starter included promoter sequence for RNA – phage T7-polymerase (Invitrogen). Then the following was added to the reaction, in this order: 4 µl of 5× First Strand Buffer, 2 µl of 0.1 M DTT, and 1 µl of 10 mM dNTPs. After 2 minutes of pre-incubation at 42°C, 2 µl (200 U) of reverse transcriptase Superscript II (Life Technologies) was added to the reactive mixture and incubation was continued for another hour. In order to obtain the second thread of cDNA, the following was added to the reaction: 30 µl of 5× Second Strand Buffer, 91 µl of RNase-free water, 3 µl of 10 mM dNTPs, 4 µl (40 U) of E. coli DNA Polymerase I (Life Technologies), 1 µl (10 U) of E. coli DNA Ligase (TaKaRa), 1 µl of (2 U) Rnase H (TaKaRa). The mixture underwent 2-hour incubation at 16°C. After that time, 2.5 µl (10 U) of T4 DNA Polymerase I (TaKaRa) was added to the mixture, and the entire mass underwent incubation at 16°C for another 5 minutes. The reaction was blocked by adding 10 µl of 0.5 M EDTA whereas the double-thread cDNA (ds cDNA) was extracted by using the phenol/chloroform method. The aqueous phase was separated using Phase Lock Gel (Eppendorf). After precipitation, cDNA was dissolved in 12 µl of water free from RNases. 10 µl of ds cDNA constituted the matrix for synthesis of biotinilised cRNA (BioArray High Yield RNA Transcript Labeling Kit, Enzo Diagnostic). The marked cRNA was purified in columns of RNeasy Mini Kit (Qiagen), fragmented and hybridized, first with test micromatrix (Test3), and then with the micromatrix of Human Genome Arrays U133A (Affymetrix). Washing, staining with streptavidyne-phycoerythrin complex and scanning of micromatrix in GeneArray (Agilent) scanner was carried out in line with the instructions of Affymetrix Gene Expression Analysis Technical Manual. The absolute and comparative analyses were carried out by means of the software Affymetrix GeneChip Analysis Suite 5.0. The results obtained after comparative analysis were then sorted to single out genes with changed expression, using the software Affymetrix Data Mining Tool.

The results were normalized using RMAExpress. Next the hierarchical clustering analysis was used for each tested group, based on Cluster v.3.0. It is an agglomerative approach in which single expression profiles are joined to form groups, which are further joined until the process has been carried to completion, forming a single hierarchical tree. There are several variations on hierarchical clustering. We used single-linkage hierarchical clustering using an Euclidean distance metric showing the differences in expression levels among analyzed measurements.

The aim of this analysis was comparison of transcriptional activity of telomerase activators and inhibitors encoding genes in CD34<sup>+</sup> cell cultures obtained from cord blood directly after collection and 24 hour after collection. Data included three CD 34<sup>+</sup> cell cultures. Cultures 1 and 2 were made of cells obtained from cord blood directly after collection and cell culture 3 – 24 hour after. CD34<sup>+</sup> cell cultures was divided additionally into two fractions: cells CD 34<sup>+</sup> adjacent (A) and not adjacent (NA) to plastic.

**Real Time PCR assay (QRT-PCR).** We performed a real-time quantitative RT-PCR assay to quantify the numbers of copies of mRNA of telomerase subunits (TERT, hTR and TP1) in CD34<sup>+</sup> cells obtained directly after childbirth and 6, 12 and 24 hours after cord blood collection. Transcriptional activity of telomerase sub-

units encoding genes is expressed as numbers of copies of a given mRNA (TERT, hTR and TP1) per 1 µg of total RNA appointed on the basis of kinetics of real-time PCR.

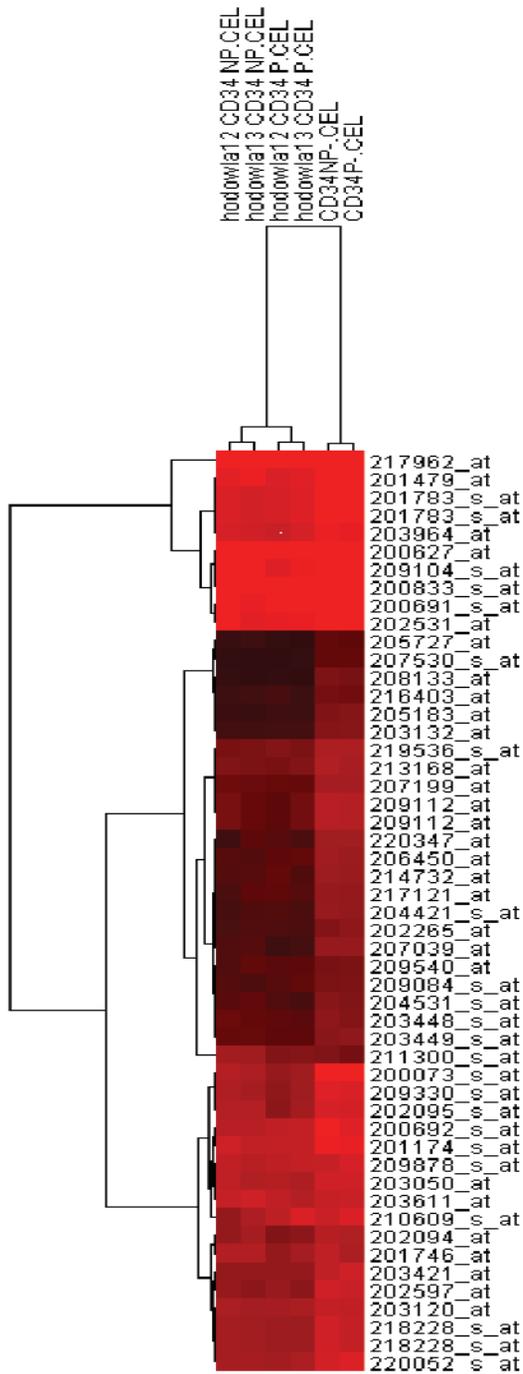
Analysis of the expression of hTERT, hTR and TP1 telomerase subunits was performed by quantitative polymerase chain reaction method based on fluorescent TaqMan methodology (ABI Prism 7000 Sequence Detection System) capable of measuring fluorescence in real-time.

Primers for amplification of telomerase subunits (TERT, hTR and TP1) were determined using the computer program Primer Express™ Version 1.0 (Perkin-Elmer Applied Biosystems, Foster City, CA) and checked by BLAST. Primers sequence was: TERT-2161 5'-CGCCGCTGAGCTGTACTTTGTTC-3' (sense), TERT-2611 5'-TTGTTCTCCATGTCGCCGTAG CA-3' (antisense) for hTERT subunit; TERT-2191A 5'-ATGTGACGGGCGCGTACGAC-3' (sense), TERT-2611 5'-TTGTTCTCCATGTCGCCGTAGCA-3' (antisense), for hTERT subunit; hTR-24 5'-AGGCGCCGTCTTTTGTCTCC-3' (sense), hTR-146 5'-GGCCAGCAGCTGACATTTTTTGT T-3' (antisense) for hTR and TP1-2769 5'-GCAGCATCCGGCTTTTCATTTCA-3' (sense), TP1-3138 5'- TGCAGACGTTGGTTCCGGTTCAG -3' (antisense) for TP1. We performed BLASTN to confirm the total gene specificity of the nucleotide sequences chosen as primers and probes. Total RNA was reverse-transcribed for single strand cDNA using Tth polymerase. QRT-PCR was performed using the ABI PRISM7000 Sequence Detection System (RT: 50°C - 30 min.; polymerase activation 95°C 15 min.; PCR: 45 cycles: 94°C - 15s, 60°C - 30s, and elongation 72°C- 10 min.). Quantified transcripts of the gene GAPDH encoding human glyceraldehyde-3-phosphate dehydrogenase (using oligonucleotide 5'-GAAGGTGAAGGTCGGAGTC-3' as forward primer and 5'-GAAGATGGTGATGGGATTC-3' as reverse primer) and β-actin (using oligonucleotide 5'-TCACCCACTGTGCCCATCTACGA-3' as forward primer; 5'-CAGCGGAA-CCGCTCATTGCCAATGG-3' as reverse primer and 5'-ATGCCCTCCCATGCCATCCTGCGT-3' as probe) gene as an endogenous RNA control were carried out. The copy number of each sample is inferred from the C<sub>T</sub> and by using the standard curve performed during the same experiment. The number of ER-alpha mRNA copies was calculated based on the calibration curve for β-actin standards performed during the same experiment. The obtained results were recalculated for 1 µg of total RNA. Primers and probe specifics were confirmed by sequence analysis using ABI PRISM 377 DNA Sequencer (PE Applied Biosystems).

**Ethical issues.** Consent for the experiment was obtained from The Research Committee for Human Subjects of The Medical University of Silesia.

**Statistical analysis.** Statistical analysis was performed with the use of STATISTICA data analysis software system (StatSoft, Inc. 2001, version 6. www.statsoft.com). Thus, the normal distribution was calculated using Shapiro-Wilk test and showed lack of such distribution, therefore, to assess differences between particular groups nonparametric test (Wilcoxon-Cox test) and parametric t Student and Welch's tests (t-test corrected for unequal variances) were employed, at the p=0.05 significance level. The dynamics of changes of analyzed parameters levels was presented graphically in figure of temporary courses. The Lilliefors and Bera-Jarque normality tests were used for nonparametric residuals. We used Grubbs' algorithm. This algorithm assumes that the finite observations of a study are taken from a Gaussian population.

Comparisons between time-intervals were made independently in subgroups, using t test or Wilcoxon-Cox test. Calculations were done with use R language (<http://www.bioconductor.org/>) as well as Matlab system (<http://www.mathworks.com/>).

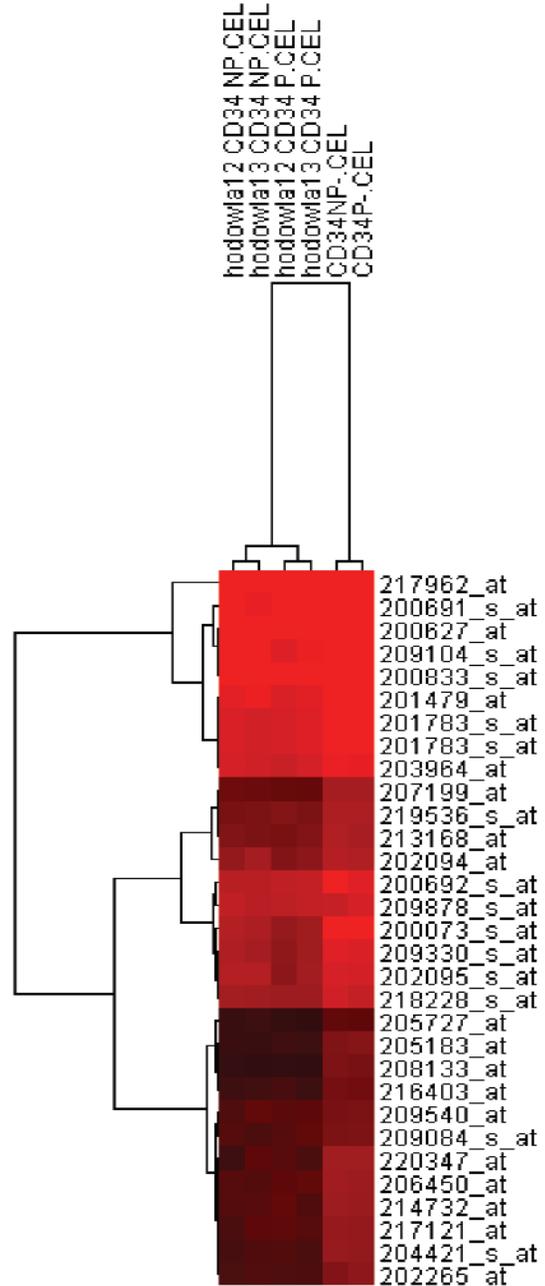


**Fig. 1.** Dendrogram for all transcripts of telomerase activators and inhibitors based on normalized fluorescence value. Genes in the demonstration data set were subjected to single-linkage hierarchical clustering using a Euclidean distance metric and gene-expression families.

## Results

### Microarray Assay

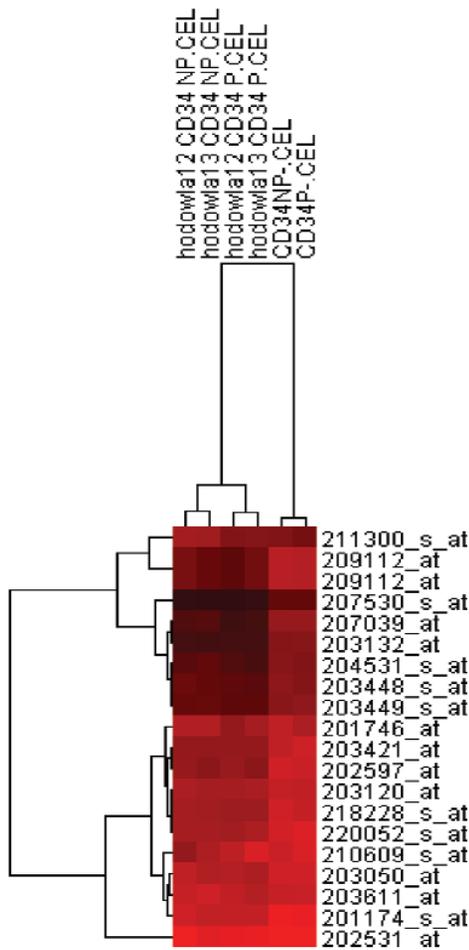
**Transcriptional activity of telomerase activators and inhibitors encoding genes.** Fig. 1 presents the obtained results of clustering. Clusterization was car-



**Fig. 2.** Dendrogram for all transcripts of telomerase activators based on normalized fluorescence value. Genes in the demonstration data set were subjected to single-linkage hierarchical clustering using a Euclidean distance metric and gene-expression families.

ried out for all transcripts of telomerase activators and inhibitors. The dendrogram shows the correlation between expression profiles of transcripts of telomerase activity regulators encoding genes.

Hierarchical clustering yielded three groups of separate clusters. The first group includes CD 34<sup>+</sup> cells adjacent to plastic. The second group consists of CD 34<sup>+</sup> cells not adjacent to plastic. And the last group includes CD 34<sup>+</sup> cells adjacent and not adjacent to plastic.



**Fig. 3.** Dendrogram for all transcripts of telomerase inhibitors based on normalized fluorescence value. Genes in the demonstration data set were subjected to single-linkage hierarchical clustering using a Euclidean distance metric and gene-expression families.

Hierarchical clustering yielded three groups of separate clusters. The first group includes CD 34<sup>+</sup> cells adjacent to plastic. The second group consists of CD 34<sup>+</sup> cells not adjacent to plastic. And the last group includes CD 34<sup>+</sup> cells adjacent and not adjacent to plastic.

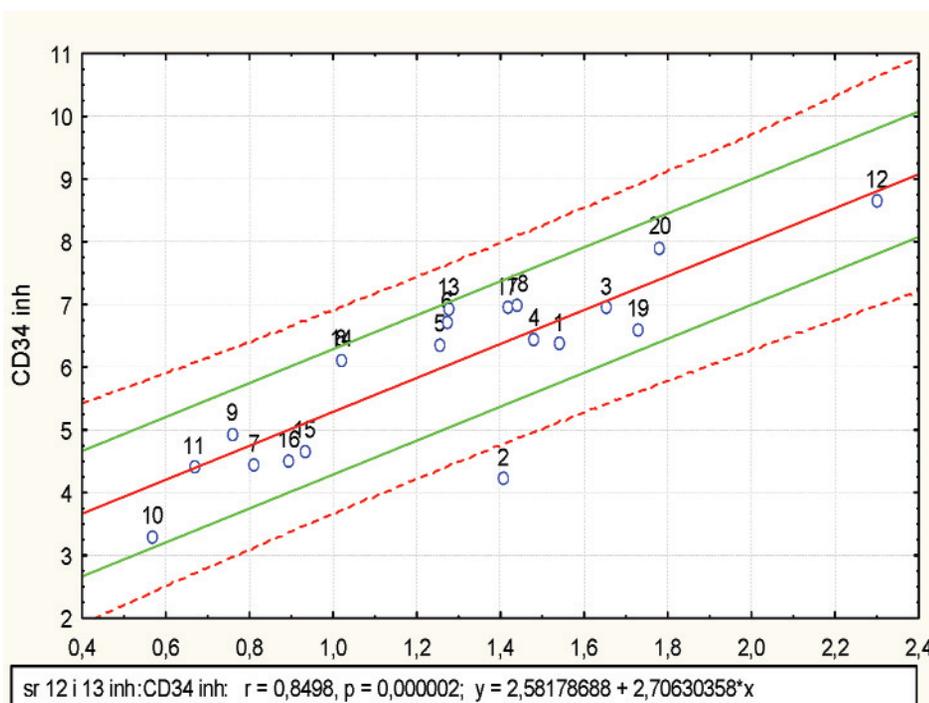
Hierarchical clustering yielded three groups of separate clusters. The first group includes CD 34<sup>+</sup> cells adjacent to plastic. The second group consists of CD 34<sup>+</sup> cells not adjacent to plastic. And the last group includes CD 34<sup>+</sup> cells adjacent and not adjacent to plastic.

**Differentiating genes of CD34<sup>+</sup> cell cultures obtained directly after childbirth and 24 hours after cord blood collection.** To identify differentiating genes from groups of telomerase activators and inhibitors, linear regression was performed. The regression model is indexed with biologically meaningful gene-specific parameters.

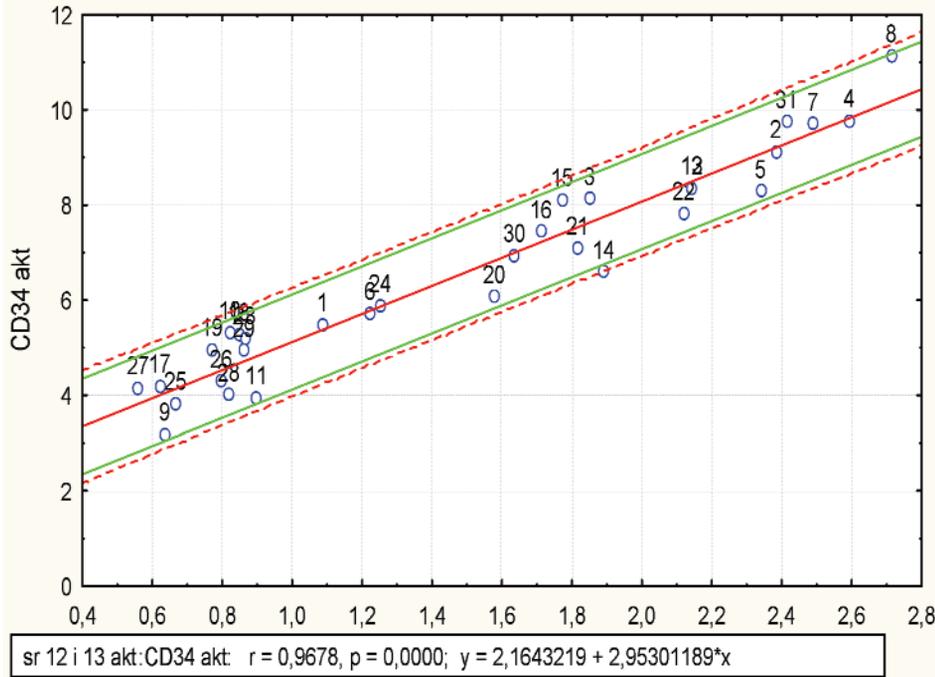
In regression analysis apart from the statistical criterion, realizing the possible delimitation round line of regression the area of prognosis is regarded as the biological criterion. The biological criterion is defined as twofold increase or decrease in value of gene expression.

The statistical criterion (the prognosis area) implicates the biological criterion: differentiating gene: (*TERT* – telomerase reverse transcriptase; ID:207199\_at) (Fig.4) are located above the statistical criterion.

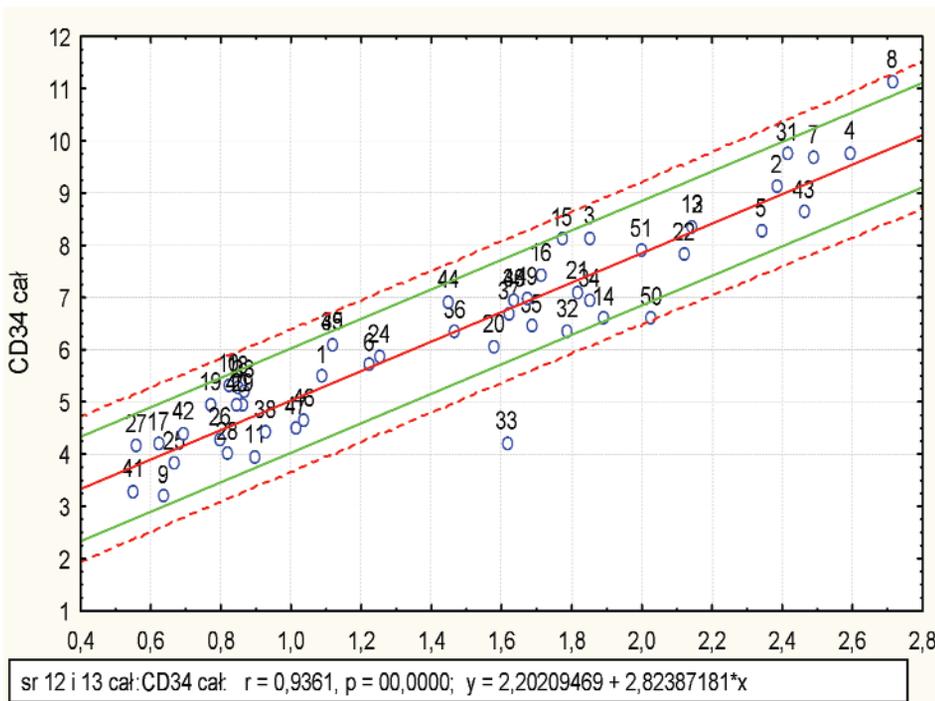
The statistical criterion (the prognosis area) implicates the biological criterion: differentiating gene: (*RELA* – reticuloendotheliosis viral oncogene homolog A; ID: 209878\_s\_at) (Fig.5) are located on border of statistical criterion.



**Fig. 4.** Regression analysis for normalized fluorescence value of telomerase inhibitors transcripts in CD34<sup>+</sup> cell cultures obtained directly after childbirth and 24 hour after cord blood collection.



**Fig. 5.** Regression analysis for normalized fluorescence value of telomerase activators transcripts in CD34-cell cultures obtained directly after childbirth and 24 hour after cord blood collection.



**Fig. 6.** Regression analysis for normalized fluorescence value of telomerase regulators transcripts in CD34-cell cultures obtained directly after childbirth and 24 hour after cord blood collection.

The statistical criterion (the prognosis area) implicates the biological criterion: differentiating gene: (*P53* – tumor protein P53; ID: 211300\_s\_at) (Fig.6) are located above statistical criterion.

**Real Time PCR assay (QRT-PCR)**

**Transcriptional activity of hTR telomerase sub-units encoding gene in CD34- cells.** Transcriptional

activity of hTR encoding gene was analysed in CD34-cells obtained in different time-intervals (0h, 6h, 12h and 24h from collection) from women after spontaneous labour. The average of transcriptional activity of hTR encoding gene equaled 24567 mRNA copies in 0h; 21456 copies of mRNA in 6h; 22456 in 12h and 25467 mRNA copies in 24h.

The Levene's test for equality of variances and Wilcoxon test were used to evaluate the variation of

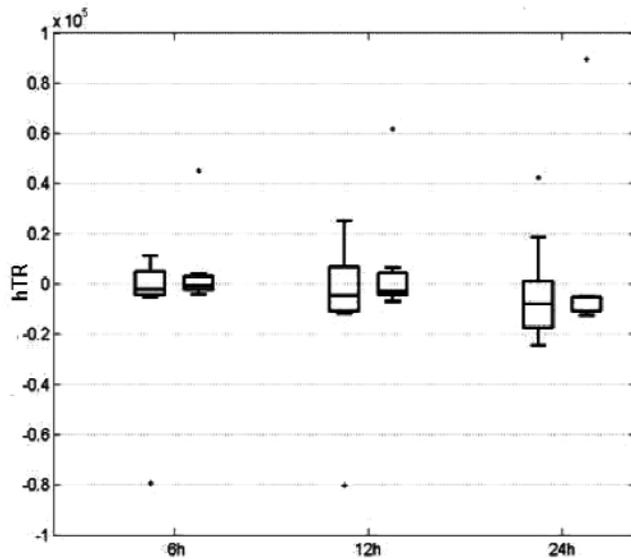


Fig. 7. Comparison of hTR mRNA copies per µg of total RNA between 0h, 6h, 12h and 24h after cord blood collection.

numbers of copies of hTR mRNA in successive time-intervals. Numbers of copies of hTR mRNA were not significantly different between all time-intervals (0h, 6h, 12h and 24h from collection) in CD34<sup>+</sup> cord blood cells obtained from women with spontaneous labour (Levene's test;  $p=0.18$ ; Wilcoxon test;  $p>0.05$ ).

**Transcriptional activity of TERT telomerase subunits encoding gene in CD34<sup>+</sup> cells.** Transcriptional activity of TERT encoding gene was analysed in CD34<sup>+</sup> cells obtained in different time-intervals (0h, 6h, 12h and 24h from collection) from women after spontaneous labour.

**Telomerase TERT A+B isoform of TERT subunits.** The TERTA+B isoform mRNA copy numbers was compared in different time-intervals. The Levene's test for equality of variances and Wilcoxon test were used to evaluate the variation of numbers of copies of TERTA+B isoform mRNA in successive time-intervals (Fig. 8). Our results revealed a significant decrease of TERTA+B mRNA copy numbers in the following time interval: 0h vs. 24h ( $p=0.008250$ ; t Student test).

**TERTA isoform of TERT telomerase subunits.** The Levene's test for equality of variances and Wilcoxon test were used to evaluate the variation of TERTA isoform mRNA copy numbers in consecutive time-intervals (Fig. 9).

There was a significant decrease of TERTA mRNA copy numbers after 24 hours of observation

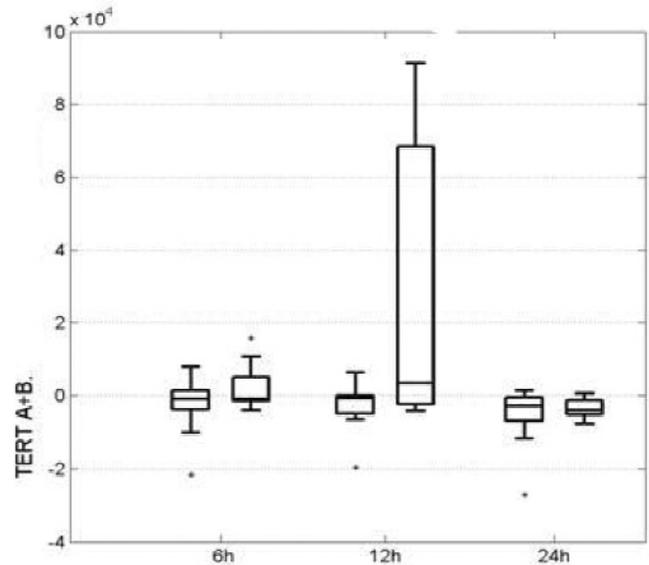


Fig. 8. Comparison of TERTA+B mRNA copies per µg of total RNA between 0h, 6h, 12h and 24h after cord blood collection.

( $p=0.002291$ ; t Student test). Changes were not observed in the first 12h.

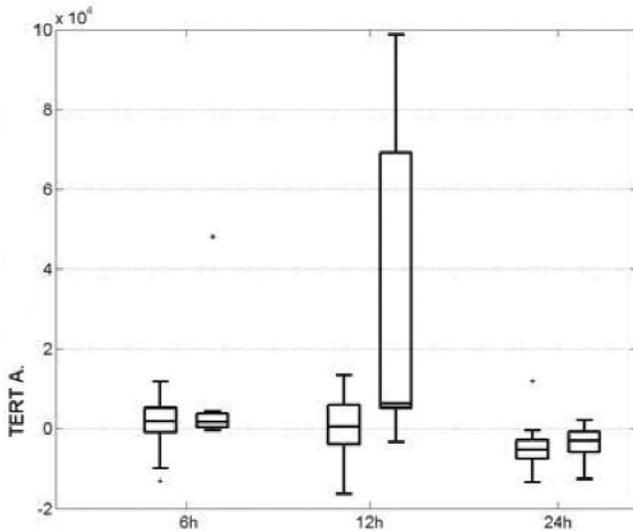
**TERTB isoform of TERT telomerase subunits.** The Levene's test for equality of variances and Wilcoxon test were used to evaluate the variation of TERTB isoform mRNA copy numbers in consecutive time-intervals (Fig. 10).

We observed significant increase of TERTB mRNA copy numbers after 6 hours of observation ( $p=0.005788$ ; t Student test; 0h vs. 6h). This level was similar after next 6h. The decrease of TERTB mRNA copy numbers occurred after 12 hours from blood collection ( $p=0.000021$ ; t Student test). The significant lower TERTB mRNA copy numbers was observed after 24h from childbirth ( $p=0.000082$ ; t Student test).

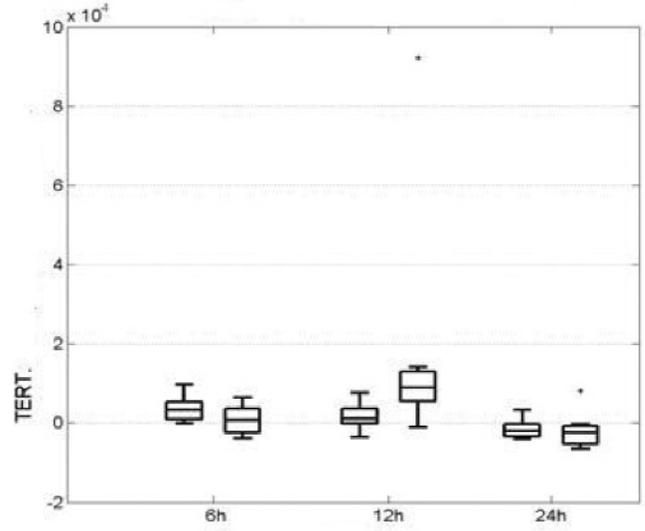
**Telomerase TERT subunits.** The Levene's test for equality of variances and Wilcoxon test were used to evaluate the variation of TERT mRNA copy numbers in next time-intervals (Fig. 11).

We observed significant increase of TERT mRNA copy numbers after 6 hours of observation ( $p=0.000439$ ; t Student test). Numbers of copies of TERT mRNA was reduced after next 6h ( $p=0.029697$ ; t test). However, it remained on a higher level than at initial moment ( $p=0.036305$ ; t Student test). The significantly lower TERT mRNA copy numbers was observed after 24h from childbirth ( $p=0.008115$ ; t Student test).

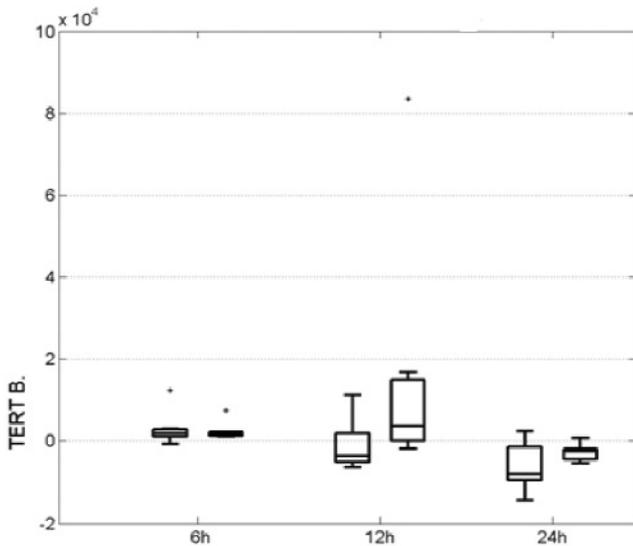
**Telomerase TP1 subunits.** Transcriptional activity of TP1 encoding gene was analysed in CD34<sup>+</sup> cells



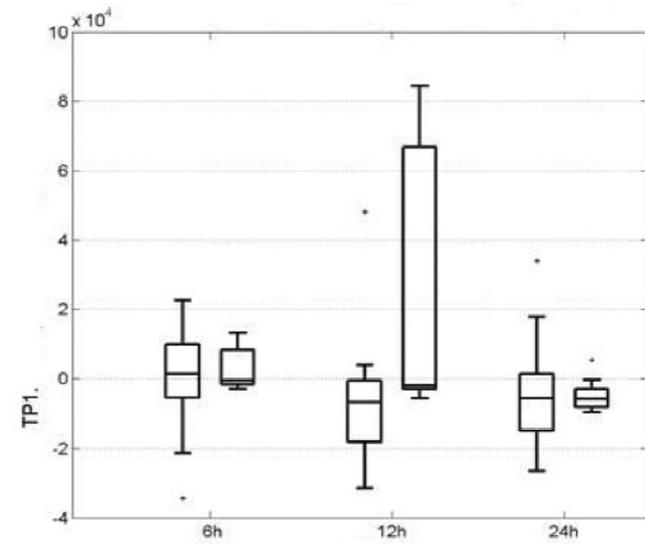
**Fig. 9.** Comparison of TERT A mRNA copies per  $\mu\text{g}$  of total RNA between 0h, 6h, 12h and 24h after cord blood collection.



**Fig. 11.** Comparison of TERT mRNA copies per  $\mu\text{g}$  of total RNA between 0h, 6h, 12h and 24h after cord blood collection.



**Fig. 10.** Comparison of TERT B mRNA copies per  $\mu\text{g}$  of total RNA between 0h, 6h, 12h and 24h after cord blood collection.



**Fig. 12.** Comparison of TP1 mRNA copies per g of total RNA between 0h, 6h, 12h and 24h after cord blood collection.

obtained in different time-intervals (0h, 6h, 12h and 24h) from women after spontaneous labour.

The Levene's test for equality of variances and Wilcoxon test were used to evaluate the variation of TP1 isoform mRNA copy numbers in next time-intervals (Fig.12).

Significant increase of number of copies of TP1 mRNA was observed between 0h and 12h after cord blood collection ( $p=0.038607$ ; Wilcoxon test). After 24h TP1 mRNA copy number did not differ significantly from initial value. The biggest change in TP1 mRNA copy number was observed between 6h and 12h ( $p 0.013064$ ; Wilcoxon test).

## Discussion

Latest research give evidence that telomerase can be regarded as an enzyme of immortality not only because it counteracts the natural process of aging (by inhibiting the telomeres shortening) but also because expression of that enzyme prevents apoptosis, normally caused by factors damaging cells. Although the mechanism of that action has not been fully revealed yet, there are many reports showing that induced overexpression of telomerase in various types of cells (including ESC) enables them to survive the genotoxic stress and facilitates cell culturing [1-9]. Other papers show that expression of telomerase increases when stress in

inflicted upon some types of cells [10-12]. Hence, there are reasons to believe that the increase of expression of telomerase is a natural response of certain types of cells to unfavorable environment which otherwise could lead to apoptosis. Such response can be treated as cells defense against apoptosis.

Certainly, one of such harmful factors, to which stem cells may be subjected and which can decrease the number of stem cells obtained from cord blood is hypoxia. Though some research report that hypoxia (3% or even 1% concentration of oxygen; in comparison to 14% in arterial blood and 5-7% in bone marrow) has a positive influence on stem cell culturing and expression of telomerase [13,14], we cannot assume that in the course of preparation of cord blood the concentration of oxygen does not fall below the critical values, harmful to stem cells.

However, the rise of telomerase expression might have been also induced by response of the whole newborns body to perinatal stress. Vaginal delivery is certainly a stress situation for the newborn and it is confirmed by works comparing physiological delivery and delivery by cesarean section. Such papers show higher level of cortisol in cord blood after vaginal delivery than after cesarean section [15-17]. Vogl *et al.* showed that levels of adrenalin, noradrenalin and ACTH are also elevated in vaginal deliveries in comparison to cesarean sections. Additionally, Malamitsi-Puchner *et al.* [18] found out that concentrations of some cytokines (IL-1beta, IL-6, TNF-alpha, IFN-gamma) are higher in cord blood and peripheral blood of the newborns delivered vaginally than by cesarean section. Interestingly, other authors [19,20] correlated one of those cytokines (IL-6) with markers of perinatal stress such as cord blood pH, higher number of NRBC or elevated concentration of cortisol. IL-6 is in turn a factor which is thought to induce the expression of telomerase [21-26]. Basing on that information, it is probable that the increase of telomerase expression could be also caused by the perinatal stress mediated by cytokines.

Taking the above mentioned into consideration, the rise in telomerase expression after 6 hours from collection observed in our work does not seem to be a desirable event. Being cells protection against apoptosis such rise proves that stem cells environment after cord blood collection is unfavorable. Similar conclusion can be drawn from papers reporting decreasing number of CD34<sup>+</sup> stem cells (Nakagawa) [27] as well as CD34<sup>-</sup> stem cells (Bieback) [28] with time from cord blood collection.

However, our results may give evidence that immortalization potential of MSC is present and is not impaired until the sixth hour from collection. Moreover, our results show that telomerase activity changes comparably fast, accurately reflecting cells actual con-

dition. Hence, it suggests that telomerase could be a good marker of stem cells "quality" expressed as an ability to inhibit apoptosis and so as an ability to sustain stress inflicted on a cell.

Regardless to causes of the rise of telomerase expression, its fall undoubtedly marks the critical moment when cells ability to sustain stress comes to an end. It also proves that 24 hours preparation time is long enough to significantly impair stem cells metabolism. The decrease in stem cells count with longer time of preparation described by Nakagawa and Bieback suggest that the process of preparation is unfavorable for the cord blood stem cells. Such decrease may be explained by the above mentioned depletion of stem cells compensation mechanisms one of which could be the ability to express telomerase. Bieback reported that no stem cells were isolated from cord blood samples which preparation time exceeded 15 hours. In our, so far unpublished, results of analysis of cord blood collected from deliveries by cesarean section, the fall of telomerase expression in MSC occurs 12 hours after collection. Concurrence of those two observations seems to confirm that impairment of stem cells ability to express telomerase leads to their apoptosis and subsequent decrease in their number. Taking that into consideration there are reasons to believe that preparation process of cord blood collected during physiological labour should finish before the fall of expression of telomerase revealed in our study, namely before 6 hours from collection. Longer time leads to decrease of MSC count and impairment of stem cells "quality".

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