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NLR's Analogs with Young Blood Cells in Monitoring of Toxicity of Long-Term Preventing Immunosuppression in the Liver Transplant's Recipients

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Abstract

The blood neutrophils to lymphocytes ratio (NLR) reflects the physiological homeostasis between lymphopoiesis and myelopoiesis, and its elevation serves as a harmful sign in many pathologies, partially, late rejection of allograft. The stem and young lymphoid cells have regenerative-trophic properties, which can affect the relevance of NLR, being opposed to immune properties, associated with bulk lymphocytes. In the present article, we have analyzed for the first time the applicability of NLR's analogs with stem and immature blood cells for monitoring harmful long-term shifting from lymphopoiesis to myelopoiesis in transplant's recipients received conventional immunosuppressive treatment. In opposition to conventional NLR, the ratio of subpopulation of CD31 cells committed to the liver tissue by alfa-fetoprotein (AFP), seems sensitive enough for such monitoring several years after transplantation of the liver from the dead.

Keywords

Liver, Transplantation, Health, Late Period, Monitoring, Stem, Progenitor Cells, NLR

1. Introduction

In the last decade, the neutrophil-to-lymphocyte blood ratio (NLR) has been evaluated as a simple and universal criterion for the severity of various pathological conditions of a person. An increase in the number of neutrophils in the N/L reflects inflammation, while lymphopenia is associated with somatic ex-

haustion (malnutrition syndrome) and hypocellularity of hematopoietic tissue [1]. The NLR competes with the "immunological jungle", for example, at selecting patients promising for transplantation [2]. NLR measured during 12 months after liver transplantation (LT) predicts overall survival in the next 7 - 9 years and closely correlates with nutrition's markers [3].

The population of human lymphoid precursors, migrating from the bone marrow to the thymus gland and peripheral blood, can affect the prognostic properties of NLR in a special, non-immune way [4]. Previously, we showed the ability of Thy-1 stage thymocytes (markers: deoxynucleotidyl transferase TdT+, CD133+, CD90+, CD34+, CD31+) to participate in regeneration [5]. Trophic influence, in particular, may answer the question of why a decrease in tissue renewal in the denominator of the ratio leads to a worse prognosis for a transplant, although lymphopenia weakens its immune rejection according to conventional wisdom.

In view of this, an ability of lymphoid progenitors CD133 and young CD31 "regulatory cells" (T reg) to become committed/aimed to the liver tissue presents additional interest [6] [7] [8] [9]. Thus, stem cells and early precursors express properties opposite to the supervisory ones. The contribution of such morphogenic-trofic lymphoid component to the prognostic properties of NLR should be studied at the level of cells in the early stages of the differentiation's continuum discussed in [10]. We tested this approach/principle for monitoring long-term periods after liver's transplantation with the aim to justify the questionable limitation of immunosuppressive treatment in it. Thus, the report is devoted to the study of dynamics of N/L with cells of varying degrees of differentiation/maturity in recipients of the liver from dead donors.

2. Methods

2.1. Patients

We report 22 liver transplant recipients cured and investigated in the department of transplantation and stem cells of the Russian Center for Radiology and Surgical Technologies named after A. M. Granov (FSBI RNCRSH, Saint-Petersburg, RF). They were regularly examined during the entire follow-up period, performing clinical and biochemical blood analysis, abdominal ultrasound with elastometry, monitoring of the concentration of tacrolimus in the blood, and maintaining its concentration at the level of 3 - 5 ng/ml. Based on the clinical laboratory data, the average LNR calculated 1, 3, 5 and 10 years after liver transplantation (LT) were not statistically different.

2.2. Blood Samples for Flow Cytometry

Blood samples 7 - 8 ml obtained at various times after surgery and were used on the day of receipt, without storage. The viability of mononuclear cells (MNC) from the entire interphase zone of the Ficoll density gradient controlled using a trypan blue exclusion test. Before cytometric phenotyping, cells were stained ac-

cording to standard procedures to detect forms in the synthetic (S) and mitotic phases (M) of cell cycle [11] with Hoechst 33342 reagent (bisbenzimidazole fluorochrome; Sigma-Aldrich, St. Louis, Missouri, USA). CD133+, CD31+ cells, CD133+ AFP+, and CD31+ AFP+ double positive cells were stained using standard Miltenyi Biotec protocol for CD133/2 antibodies (Ab) conjugated with allophycocyanin (APC), BD Bioscience Pharmingen protocol for CD31 Ab with conjugated with fluorescein isothiocyanate (FITC), and R&D Systems protocol for α-fetoprotein (AFP) Ab conjugated with phycoerythrin (PE).

2.3. Flow Cytometry

The percentage of positive cells was calculated by subtracting the value for antibodies of the corresponding control isotype. At least 500,000 total events were recorded twice to detect CD133+ cells. A dot graph of Hoechst 33342 radiation in blue (x-axis) and red (y-axis) wavelengths was used to separate the events of (G0 + G1), S, and (G2 + M) phases.

2.4. The Original Data of the Tissues' Content of the Transcripts, Which Are Typical for Different Blood Cells

Primary data on the content of transcripts of cells of various histotypes in human liver tissue and other organs were extracted from the database of free access [12] and were subjected to graph-statistical analysis.

2.5. Statistical Analyses

Individual parameters were evaluated statistically with the calculation of the mean value, standard deviation (SD) and standard error (SE). The average values of M were compared using the ***t***-test and the probability of p. The trends of parameter relationships using mathematical functions automatically generated in Excel are described. The determination of the coefficient R^2 was used as a statistical measure of the correspondence of the regression line to the data entered into the program. Satisfactory values of R^2 were confirmed using Equation (1) for the parameter t:

$$t = R^2 \times (n-2) / (1-R^2) \quad (1)$$

3. Results

3.1. General Characters of Patients during Early and Late Period after Surgery

According to **Figure 1**, the shifts of maximum of distribution (triangles) on the time's scale from "−SD" to "+SD" indicate the result of patients' selection by death between early time (0 - 16 months) and late average time (58.8 months; 24 - 106) after LT.

A shift of distribution of lymphocytes to the negative SD in a late period means a worst quality of health in the rest of long-lived persons. Most likely, the dead were those who had a lowered level of lymphocytes in the early period after

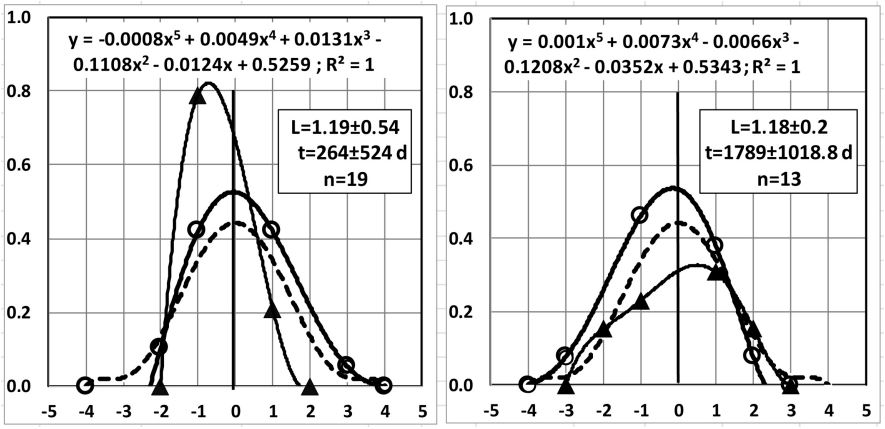

Figure 1. Distribution of individual times (t, triangles) and lymphocytes (L, circles) of recipients in the early and late periods after liver transplantation. The X-axis is the standard errors (SD) for t and L parameters. The Y-axisis the distribution of SD for t (thin lines) and L (thick lines) parameters. The dotted line is a normal distribution as a referent.

LT. Meanwhile, the rest of alive with elevated lymphocytes (left circles at −2 SD and +3 SD) acquired steadily a lymphopenia in the late period (right circles at −3 SD and +2 SD). The equality of low lymphocyte levels in early and late groups (1.18×10^9 and 1.19×10^9 per l) is a result of described redistribution of patients between average 9 and 59 months after LT.

The percentage of MNC in S-phase of the cycle increases with time after LT, meanwhile the percentage of mitotic cells (M) decreases (**Figure 2**).

3.2. Post-Operative Kinetics' Characters of the NLR's Analogs

1) To make a choice of a cell markers (m), which most interesting for investigation NLR at the lower level of cell differentiation, the original marker's transcripts "m" in the normal liver tissue (Lt) were extracted from database [12] and transformed into ratio $r = m_{Lt}/m_{BM}$, where (BM) is a normal bone marrow's tissue as a referent one. The range of such normalized (relatively) content "r" for each cell's markers let to compare them quantitatively inside an organ and between organs. The comparison of calculated "r" is shown in **Figure 3**.

If placenta (P) contains the substances provided the regeneration at large, then the markers of myeloid cells CD33, T-lymphocytes CD2, CD3, CD7, and B-lymphocytes CD19, CD27 seem less important for tissue's renewing in comparing with Thy-1, CD31, CD133, CD34, CD25, PD-1L, and DNTTIP1 ($p < 0.01$). A similar conclusion is actually for liver tissue: Thy-1, CD25, CD34 and PD-1L CD31, CD133, DNTTIP1, and CD2. Their relative content is ≥0.4 of that bone marrow ($p = 0.005$). The domination in liver and placenta of unmatured cells' markers shows the lack of understanding of the interplay between hematopoietic stem cells (HSCs) and the immune system in a liver pathology and in other pathologies as well. The morphogenic-trophic properties of unmatured lymphocytes oppose to function of security and immune surveillance associated with matured lymphoid cells [13]. Thy-1 (CD90) is a lymphoid stem cell marker,

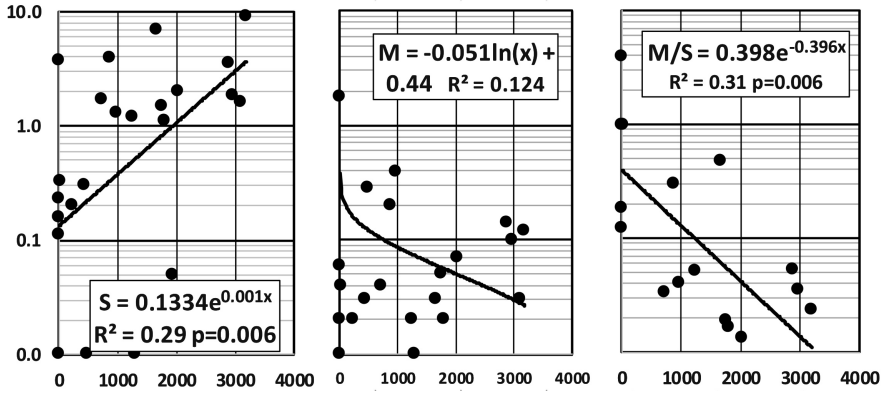

Figure 2. Proliferative activity of MNC by time (days) after LT. The approximations of the data in **Figure 2** show an exponential loss of cells reproduction. Thus, there is evidence for a reliable contribution of bulk lymphocytes in the increment of NLR during the post-operating time.

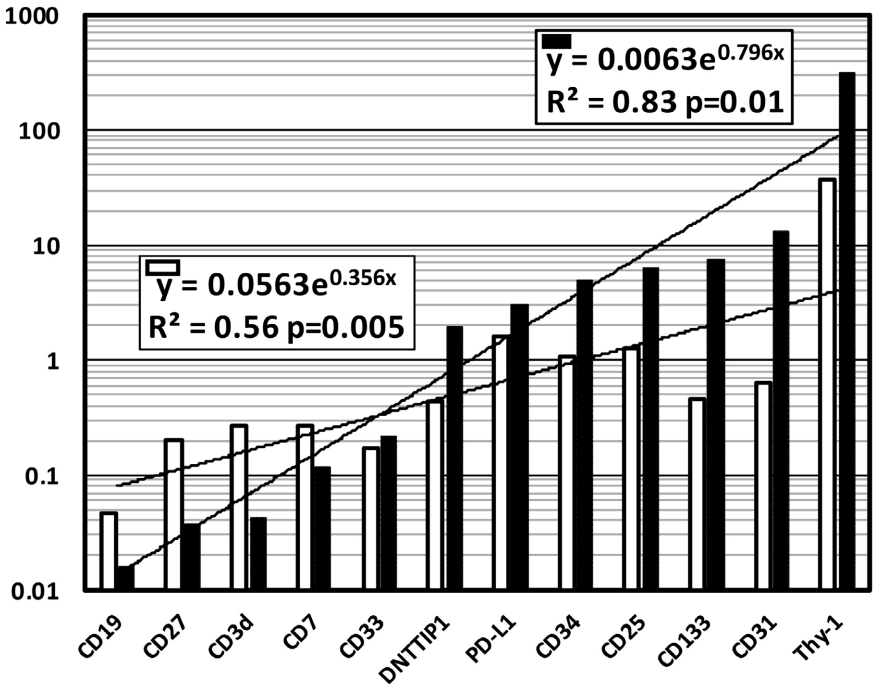

Figure 3. Distribution of relative protein markers (r) for normal liver (m_{Lt}/m_{BM}, white) and normal placenta (m_P/m_{BM}, black).

being a liver stem cell marker as well [14].

Double-positive CD34 and CD133 stem cells give rise both the early endothelial progenitors and stem cells lymphoid lineage with marker terminal deoxynucleotidyl transferase (TdT), meanwhile CD34 stem cells produce mostly erythro-myeloid progenitors [15] [16] [17] [18]. CD31 marker of T-reg and endothelial progenitors [19], and CD25 marker of T-reg lymphocytes [16] coexpress both with CD34 stem cells, and each other [6] [10] [20] [21]. Programmed death-ligand 1 (PD-L1, CD274) plays a major role in suppressing the immune system in certain cases, such as pregnancy, tissue transplantation, autoimmune

diseases, hepatitis, etc. [12]. PD-L1 expression on circulating CD34 hematopoietic stem cells closely correlated with apoptosis of Tcells [22]. Apoptosis followed by delivering of TdT in intercellular media [5]. Deoxynucleotidyl transferase terminal interacting protein (DNTTIP1), enhances the activity of terminal deoxynucleotidyl transferase TdT and vasoreparative properties of CD34 cells [12] [23].

This short overview leads us to investigate the prognostic value of NLR during the late period after LT in terms of CD133 and CD31 blood cells. The marker alfa-fetoprotein AFP used also, basing on our previous experience with it [7].

2) The average blood's content of neutrophils, lymphocytes, and subpopulations CD133, CD133 AFP, CD31, CD31 AFP in them presented in **Table 1** for the average early and late periods after LT.

According to **Table 1**, a double positive protein CD31AFP only gives a better result than conventional LNR with white blood cells (WBC). $LNR_{CD31AFP}$ results from the arithmetic division of the percentage of these subpopulations in granulocytes and lymphocytes fractions selected on the flow cytometry plot (side scattering vs. forward scattering). According to **Table 1**, the deviation of the $LNR_{CD31AFP}$ is higher (21.7-folds vs. 2.6 folds) and better statistically ($p = 0.003$ vs. $p = 0.04$) in comparing with conventional NLR with white blood cells.

Kinetic features of conventional NLR, NRL's for CD133, CD133AFP, and NLR for CD31, CD31AFP shows **Figure 4**.

Figure 4 shows unsuitability of conventional WBC's NLR for monitoring its late dynamic. The early abrupt fall of the NLR_{CD31} and $NLR_{CD31AFP}$ during 16 months after LT, followed by their slow rise up to 106 months. The most reliable long-term raise with doubling period 1.46 years found for $NLR_{CD31AFP}$ ($p = 0.015$).

Table 1. Average characters of NLR for blood cells at different level of their maturation in early and late period after LT.

Conditions	Conventional WBC	CD 133	CD133AFP	CD31	CD31AFP	Time after operation, days
N, % early	5.27 ± 0.83	0.72 ± 0.145	0.308 ± 0.096	59.04 ± 6.36	57.33 ± 6.44	89 ± 38* 29.7 ± 9.9
N, % late	3.1 ± 0.42	0.257 ± 0.043	0.527 ± 0.428	12.83 ± 2.01	10.72 ± 2.1	1891 ± 227* 1937 ± 261
p	**0.04**	**0.008**	0.62	**<0.001**	**<0.001**	**<0.001**
NLR early	6.8 ± 1.83	13.85 ± 3.78	48.9 ± 22.57	10.9 ± 4.97	229.86 ± 60.65	89 ± 38* 29.7 ± 9.9
NLR late	2.6 ± 0.39	7.97 ± 2.57	44.81 ± 11.74	2.89 ± 0.79	10.58 ± 3.44	1891 ± 227* 1937 ± 261
p	**0.04**	0.21	0.53	0.13	**0.003**	**<0.001**

(*) Average time relates to conventional data received with WBC.

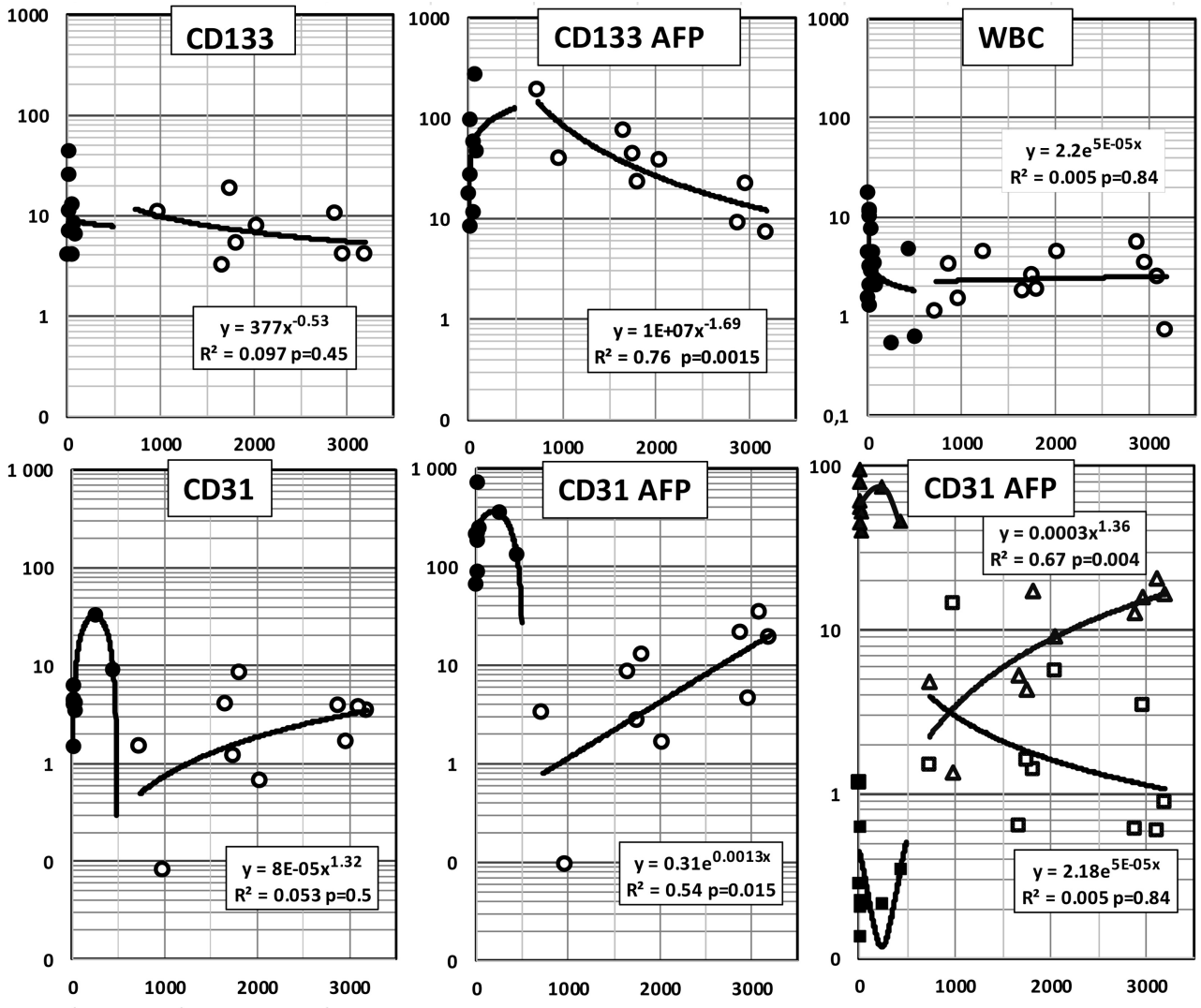

Figure 4. Long-term kinetic of the markers in the blood after LT. The X-axis is the time elapsed after the operation, days. Black symbols show early period; white symbols show late period after transplantation. The Y-axis is the value of the NLR (circles) for different populations shown in the small upper boxes. Triangles-granulocytes, %; squares-lymphocytes, %.

In parallel, calculating the doubling period of a numerator (granulocytes CD31AFP) was ≈2.37 years and the period of twofold reduction of the denominator (lymphocytes CD31AFP) was ≈3.79 years.

3.3. Discussion

The stead lowering of lymphopoiesis according to S and M/S parameters accompanied followed by low sensitivity of conventional NLR with WBC (**Figure 4**) led us to investigate the ratio with small subpopulations unmatured cells. According to **Figure 4**, period 1.2 years after LT seems optimal for selection of recipients that are most resistible to a rejection. During selection the survival can reduce to 73 percentage [24]. In result, most interesting AFP-positive CD31 subpopulation have highest CD133AFP lymphocytes and lowest both CD31AFP granulocytes and $NLR_{CD31AFP}$ at 1.9 years after LT (**Figure 4**). In relation to liver

tissue, such a shift may indicate the prevailing of capillarization over endothelial fenestration, fraught with portal hypertension [25]. Nevertheless, the main benefit of cytotoxic treatment appears to this time, *i.e.* during the early period shown by black symbols. During the next 7 years, the patients' survival decreases slowly, approximately 2.1% - 2.3% per year [26] [27], and $NLR_{CD31AFP}$ is getting worse steadily, increasing from 1 to 20 (**Figure 4**). The increase of $NLR_{CD31AFP}$ is, obviously, a more sensitive indicator than conventional NLR with WBC. CD31 lymphocytes, AFP-committed to the liver tissue, belong to immature Treg, and their slow depletion becomes toxic for recipients' alive. Thus, the $NLR_{CD31AFP}$ can be a potential measure of the harm to the health of a recipient after 1.4 - 1.9 years, with doubling time 3.79 years (**Table 1**).

There is a parallel between the prognostic advantage of NLR test with young blood cells and domination of immature lymphocytes' protein markers in the liver tissue (**Figure 3**). An increased number of subpopulations of "T-regulatory cell" (T-reg) in both the blood and in the liver transplant do not support the generally accepted immune explanation of the role of lymphopoiesis in physiology and pathology of mammals [28], partially, an "operational tolerance", a phenomenon that is believed to occur due to suppression of the immune rejection by Treg. A recent approach to reduce donor-specific alloreactivity to liver transplant, using a regulatory T-cell (Treg) as a specific immunosuppressive subpopulation [29], meets a logic objection. The studies have demonstrated that the number and suppressive activity of Treg increase with age [30]. It is not clear how these changes reconcile with worse results of liver transplantation in the elderly [31], especially, with the facts, that the risk of graft rejection is inversely related to age [32].

A reliable reason for the worse survival of an elderly after LT is their worse health rather than increased Treg as such [33]. Almost all T-reg cells are intermediate maturing cells in the differentiation's range: "hematopoietic stem cells (HSCs)/precursors of T–lymphocytes and mature/senescent T cells". Thus, an increase in "T-reg" means a "shift to the left" of the entire poorly differentiated pool, including an increase in HSCs and lymphoid precursors such as cortical thymocytes Thy, CD25, CD31 and others. Such a shift is accompanied by inevitable lymphopenia with a corresponding effect on the NLR index. This fundamental property of lymphocytopoiesis is ignored by the dominant immunological interpretation of the causes of operational "tolerance", despite the ever-increasing amount of information about the beneficial effect of HSC and early (immature) precursors on the regenerative functions of various organs and tissues in various pathological situations. Such a "feeder, trophic, morphogenic" ability of immature lymphocyte and HSCs can mimic the phenomena described as immune evasion in cancer or operational "tolerance" in organ transplantation, and many other physiological/pathophysiological phenomena that are today in the field of purely immunological interpretations, in particular, suppression of immunity by "regulatory" cell. In the view morphogenic properties of

young lymphocytes, the oppose dynamic lymphoid and myeloid components of CD31AFP cells reflects the interference of symmetric hematopoiesis of white blood cells with asymmetric (normal) one. It stays a maximal during 1.2 years after LT, normalizes to a minimal at 1.9 years, and increases repeatedly toward 8 - 9 years after LT. These referent periods correspond to dying of originally weak recipients, selection of ≈70% of the alive /benefit, and getting them slowly to the worst, probably, via secondary decapillarization (**Figure 4**). A normal liver belongs to the group of tissues with the lowest original/natural vascularization in terms of endothelial markers CD31 in tissues, and with the shortest life span in case of their malignization [13].

Thus, the strategy of recipients' care after 1.4 - 1.9 years has to keep under control the achieved lowest $NLR_{CD31AFP}$ by reduction of the immunosuppressing therapy, as it diminishes of CD31AFP' lymphocyte content in the blood.

4. Conclusion

In opposition to conventional NLR, the ratio of subpopulation of CD31 cells committed to the liver tissue by AFP, seems acceptable for monitoring of harmful long-term shifting from lymphopoiesis to myelopoiesis in a transplant's recipients received conventional immunosuppressive treatment.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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