***Original article***

# Childhood Acute Lymphoblastic Leukemia: Detection of Minimal Residual Disease after Allogeneic Transplantation

## Leucemia acută limfoblastică la copii: detecția bolii minime reziduale după transplantul alogeneic

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### Abstract

*Most minimal residual disease-directed therapeutic interventions in current treatment protocols for acute lymphoblastic leukemia (ALL) are based on bone marrow and peripheral blood testing. In this study, we employed real-time quantitative polymerase chain reaction analysis (Real Time PCR) to examine minimal residual disease (MRD) in 28 patients (pts) with ALL, 36% receiving transplantation from related donors and 64% receiving transplantation from nonrelated donors. Determined MRD positivity ranged from positive below quantitative range (2 pts, 7.17%), between 0.01 % and 0.1 % (1 pts, 3.57%), and higher than 0.1 % (6 pts., 21.42%). Eight patients were MRD negative and during observation the MRD became positive. Two patients determined to be MRD positive (0.0001 %) in the early phase showed subsequent increase of MRD load, while another two, who were determined to be positive in the later phase did not. We had two patients with strong positive MRD in which during observation the MRD turned to negative due to clinical intervention. However, for 5 patients with high MRD load the induction of remission was not obtained. As a perspective, a well characterized and homogenous patient cohort should be enrolled in a clinical study to investigate the impact of post transplant MRD on the outcome.*

***Keywords:*** *acute lymphoblastic leukemia, peripheral blood, bone marrow, minimal residual disease*

### Rezumat

*Cele mai multe dintre intervențiile terapeutice direcționate către boala minimă reziduală in protocoalele curente de tratament pentru leucemia acută limfoblastică (LAL) sunt bazate pe testarea măduvei osoase şi a sângelui periferic. În acest studiu, s-a utilizat metoda de analiză cantitativă a reacției de polimerizare în lanț în timp real pentru a examina boala minimă reziduală la 28 de pacienți cu leucemie acută limfoblastică (LAL), 36% primind transplant de la donori înrudiți şi 64% primind transplant de la donori neînrudiți. Pozitivitatea MRD determinată variază de la domeniul de cantitație pozitiv scăzut (doi pacienți, 7,17%), între 0,01 % şi 0,1 % (un pacient, 3,57%), şi mai ridicat de 0,1 % (şase pacienți, 21,42%). Opt pacienți au avut rezultatele MRD negative şi în timpul observațiilor MRD a devenit pozitiv. Doi pacienți, ce au avut MRD pozitiv (0,0001 %) în faza inițială, au arătat o creştere ulterioară a MRD, în timp ce alți doi pacienți, ce au fost pozitivi în faza târzie nu au*

*arătat o creştere ulterioară a MRD. Au fost doi pacienți cu MRD puternic pozitiv şi în timpul observațiilor MRD a devenit negativ datorită intervențiilor clinice. Totuşi, pentru cinci pacienți cu MRD ridicat nu s-a obținut inducerea remisiunii. Ca şi perspectivă, ar trebui să se facă o caracterizare a unui grup omogen de pacienți ce sunt introduşi într-un studiu clinic în vederea investigării impactului post transplant al MRD asupra rezultatelor.*

***Cuvinte cheie:*** *leucemia acută limfoblastică, sânge periferic, măduvă osoasă, boală minimă reziduală*

## Introduction

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is an effective post-remission therapy in patients with ALL, but it is associated with significant toxicity, so the optimal timing and use of this modality remains an issue of debate. Increased advances in reduced-intensity transplant preparative regimens and alternative donors have increased the accessibility to allogeneic transplantation (1, 2). A risk adapted paradigm, using minimal residual disease analysis, may help in the selection of patients at highest risk for relapse, who may benefit most from alloHSCT (3, 4).

Minimal residual disease is the name given to small numbers of leukaemic cells that remain in the patient during treatment, or after treatment when the patient is in remission (5, 6). MRD is the major cause of relapse in cancer and leukaemia (7, 8). Nowadays several very sensitive molecular biology tests, based on DNA, RNA or proteins are available, though these types of methods can measure levels of cancer cells in tissue samples, sometimes as low as one cancer cell in a million normal cells. In cancer treatment, particularly leukaemia, MRD testing has important roles, such as determining whether treatment has eradicated the cancer or whether traces remain, comparing the efficacy of different treatments, monitoring patient's remission status and recurrence of the leukaemia or cancer and choosing the treatment (9, 10). The tests are not simple, are often part of research or trials, and some have been accepted for routine clinical use (11- 13).

Most minimal residual disease-directed interventions in current treatment protocols for acute lymphoblastic leukemia are based on bone marrow testing, which is a consequence of previous studies showing the superiority of bone marrow over peripheral blood as an investigational material (14, 15).

In this article, we characterize the leukemia of 28 patients, establishing allele specific oligos (ASO) and measuring the MRD during their follow up period after transplantation.

## Materials and methods

### *Patient characteristics and samples collection*

Samples were collected from 28 patients treated for ALL at the Children's Hospital of the JW Goethe University, Frankfurt, Main, Germany. From a total of 28 patients, 15 patients were males, and 13 were females. The age average of patients was 10 years and 9 months, and the range age of MRD patients was between 2-23 years old. 10 patients (35.7%) received transplantation from related donors (people) and 18 patients (64.3%) received transplantation from nonrelated donors (people).

Informed consent was obtained from all subjects and the experiments performed for this investigation comply with current guidelines and ethics. Bone marrow aspirates were collected from patients, and all analysis were carried out with national and local ethical consent. All samples are collected on EDTA recipients and could be preserved a short period at 4ºC.

### *Study Population*

28 consecutive patients were analyzed for MRD at the Children's Hospital of the JW Goethe University, Frankfurt, Main, Germany. Baseline characteristics are shown in *Table 1*.

From a total of 28 ALL patients, 14 patients were found to have a common type of ALL, 2 patients were found to have B-ALL, 4 patients

**Table 1. Patients Demographics**

| Patient ID | Sex (M/F) | Immunological type of ALL | Age of Patient |
|---|---|---|---|
| 1 | M | Common | 5 |
| 2 | F | B-ALL | 4 |
| 3 | M | Unknown | 15 |
| 4 | M | Common | 12 |
| 5 | M | pre-B-ALL | 16 |
| 6 | M | Common | 12 |
| 7 | F | Common | 12 |
| 8 | F | Common | 7 |
| 9 | F | Common | 8 |
| 10 | F | B-ALL | 9 |
| 11 | M | Common | 13 |
| 12 | F | Common | 12 |
| 13 | F | Common | 4 |
| 14 | M | pre-B-ALL | 7 |
| 15 | M | pre-B-ALL | 23 |
| 16 | M | Common | 22 |
| 17 | M | pre-B-ALL | 12 |
| 18 | M | Common | 7 |
| 19 | M | Common | 19 |
| 20 | F | Unknown | 10 |
| 21 | F | Unknown | 12 |
| 22 | M | Common | 8 |
| 23 | F | pre-T-ALL | 10 |
| 24 | M | Common | 14 |
| 25 | F | pre-T-ALL | 2 |
| 26 | F | pre-T-ALL | 14 |
| 27 | M | pre-T-ALL | 13 |
| 28 | F | pre-T-ALL | 3 |

were found to have pre-B-ALL, 5 patients were found to have pre-T-ALL, 3 patients were found to have an unknown type of ALL.

***Target identification***

Detection of patient specific junctional regions of Immunoglobulin heavy chains (Ig-$VH_{1-6}$-$JH_{1-6}$), Immunoglobulin kappa light chains (Ig$V\kappa_{I-IV}$-$Jk_{1-5}$ or Ig$V\kappa_{I-IV}$-$Jk_{1-5}$-κ-deleting element) gamma T cell receptors (TCR$V\gamma_{I-IV}$-$J\gamma$), complete and incomplete delta T cell receptors (TCR$V\delta_{1-3}$-$J\delta_1$; $V\delta_2$-$D\delta_3$; $D\delta_2$-$D\delta_3$; $D\delta_2$-$J\delta_1$) and deletions (TCR$V\delta_2$-$J\alpha_{1-2}$), complete and incomplete beta T cell receptors (TCR$V\beta_{2-24}$–$J\beta_{1-2}$, $D\beta_{1-2}$-$J\beta_{1-2}$), and subsequent heteroduplex analysis were performed as described in detail (16-18).

***Nucleotide sequencing***

PCR products were sequenced after elution of the appropriate PCR product from the acrylamide gel. Sequencing was performed in both directions with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany) with the same oligonucleotides used for screening PCR reactions. Analysis was performed on a 3100 ABI automated

**Table 2. Identified rearrangements characterizing leukemia blasts at initial diagnosis**

| No. | Ig | TCR-γ | TCR-δ | TCR-β | Marker 1 and Oligonucleotide | Marker 2 and Oligonucleotide |
|---|---|---|---|---|---|---|
| 1 | IGHV6-D7-JH3 | Vg9-JP1 | VD2-DD3<br>VD2-J9<br>VD2-J29 | | IGHV6-3F01<br>CTAGGAGAACTGGGGATCCA | VD2-DD3F01<br>TGTGACACCGGGGGT |
| 2 | IGHV6-D2-JH6 | Vg11-JP1 | VD2-J9 | | Vg5-Ig1.1/2.1F02<br>CCTGCTGGATGAGACTGGT | IGHV6-6F02<br>CCAGCTGCTACGGCAC-TACT |
| 3 | IGHV6-JH4<br>IGVH4-D2-JH6 | Vg5-J1 | VD2-DD3 | | VH4-6F01<br>CTGGTGGTGTATGCTACTTTTTAC | VH6-4F01<br>CAAGAGCTGGGAGCTAG-GAC |
| 4 | IGVH4-D3-JH4<br>IGHV1-D3-JH3 | | DD2-DD3<br>VD2-J29 | | IGHV4-4F02<br>CGCGGCTCGGAAGAGAAGTAT | VD2-DD3F01<br>CCTGCCGTACGGGGACT |
| 5 | | Vg1-JP1 | VD2-KDE<br>VD2-J53 | DB1-J2-7 | Vd2JaF01<br>CTTCCCGGCCAAGCA | Vg-JP1F01<br>TGGGACFGGGCCGATAC |
| 6 | IGHV3-JH6<br>IGKV3-KDE | Vg2-J1 | VD2-J29 | | Ja9R02<br>TGAATCCTCAGCCGTGTC | Vg5-Ig1.1/2.3F01<br>CTATTACTGTGCCACG-GAGA |
| 7 | IGHV3-JH3 | | | | IGHV3-3F02<br>TGTGCGAGAGCCTCGAAG | |
| 8 | IGVH3-D6-JH4<br>IGKV1-KDE | | VD2-DD3<br>VD2-VD3 | | IGHV3-4F02<br>GCTATGGCAGCTGCTATGTAC-TAC | VKII5-KdeF01<br>CCAACAGTATAATAGT-TATTCCGTAAG |
| 9 | | Vg9-JP2<br>Vg3-JP2 | VD2-VD3 | | DD2-5DD3F02<br>GCCTTCCTAGAAGGGGATA | |
| 10 | IGHV3-JH4<br>IGKV2-KC | | | | IGHV3-4F01<br>CTCAGACAACTATGACTAT | |
| 11 | IGVH3-D7-JH4 | Vg9-J1 | | VB27-J2-4 | IGHV3-4F03<br>GTATTACTGTGCAAAAGATA-CATCT | JB2-4F01<br>CGCGACGCCGGGAG |

nucleic acid sequencer (Applied Biosystems) and electropherograms were reanalyzed by visual inspection to check for ambiguous base readouts.

***Interpretation of junctional sequences***

Variable (V), diversity (D), and joining (J) elements of the immune receptor genes were identified by comparison to known human germline alleles using the IMGT/V-QUEST (19), the BLAST (20), and IGBLAST (21) search.

Allele-specific oligonucleotides were used in combination with germ line TaqMan probes and germ line reverse primers for quantitative real time PCR (22-25). Applied criteria for determining the reliable quantitative range and the maximum sensitivity are in accordance to published guidelines of the ESG-MRD (26). To correct the MRD levels in follow-up samples for the quantity and quality of DNA, RQ-PCR analysis of the albumin gene was used (27).

## Results and Discussions

Techniques employed for MRD analysis rely on leukemia cell specific marker detection, which enables to distinguish blasts from normal marrow cells. Those leukemia cell specific markers have to be detected with high sensitivity, because of their small numbers. Markers which are currently used are genetic markers detected by PCR, and immunophenotypic markers detected by flow cytometry. It is established that quantitative MRD data have a higher predictive value than qualitative ones (6, 28-30). Sensitivity of the assay has to be accur-

*Table 2. Identified rearrangements characterizing leukemia blasts at initial diagnosis (continued)*

| | | | | | | |
|---|---|---|---|---|---|---|
| 12 | IGVH3-D6-JH4 | Vg8-JP1 | | | IGHV3-4F01<br>CGCCTTGTATTACCGGAGA | Vg-JP1F02<br>CCACCTGGGATAGAACCA |
| 13 | IGVH3-D1-JH4<br>IGVH118-JH4 | Vg8-JP1 | | | IGHV1-5F01<br>GTACCAGCTGCCAGTACTG | IGHV3-4F02<br>AGAGTGGGAGC-<br>TACTCCATAT |
| 14 | IGVH3-D3-JH5<br>IGVH3-D3-JH6 | | VD2-DD3 | | IGHV3-6F02<br>GTGTATTACTGTGCAAGGTTT | VD2-DD3F01<br>GCCTGTGACACCGGAT |
| 15 | IGVH3-D6-JH4<br>IGVL3-JL3 | | | | IGHV3-4F01<br>AACCCCAATCAGCGGAA | Vl3Jl3F02<br>CAGTGGTAACCATCAGG-<br>GTGT |
| 16 | | Vg2-J1 | | | Vg5-Ig1.1/2.3F02<br>GGGCCGGGTGGTATAAGA | |
| 17 | IGVH3-D3-JH4<br>IGVH4-JH4<br>IGKV1-KDE | Vg2-J1 | VD2-DD3 | | Vg5-Ig1.1/2.3F01<br>CCCCCTATGACCGAATGA | VKI5-KdeF01<br>TAT-<br>TATAGTTTCCCCCCGACA |
| 18 | IGVH3-D2-JH4 | | VD2-DD3<br>DD2-DD3 | | IGHV2-6F02<br>GCCCTACGCAACGACTA | IGHV3-4F02<br>GTAGTACCAGCTGCTAT-<br>TAAATAGA |
| 19 | | Vg3-JP5<br>Vg10-J2 | VD2-JD1 | VB1-J2-<br>7 | Jb2-7F01<br>TGGGGACAGGGGTGAGA | Vg5-Ig1.1/2.3F01<br>GGGACAGGCCCCCGT |
| 20 | IGVH1-D3-JH4<br>IGKV1-JL6 | Vg2-J1<br>Vg2-JP1 | | | Vg5-Ig1.1/2.3F02<br>TATGATAGTAGTGGTTACCGACT | Ja29R01<br>CTGAATTAGGCCCCCGA |
| 21 | IGKV1-KDE | | VD2-DD3<br>VD2-J9 | | Ja9R02<br>GTATCCCCCAGCCCTAG | VD2-DD3F01<br>CCTGTGACACCTAGGGCT |
| 22 | IGVH6-JH4<br>Kintron-KDE | | VD2-J29<br>VD2-J61 | | IGHV6-4F01<br>AGGCTCATAGTGGGAGCT | Ja29R01<br>CTTGGCCAACGTCCT-<br>TAACA |
| 23 | | | DD2-DD3 | | DD2-5DD3F02<br>ATTGTGCCTTCCTAGGGTG | |
| 24 | IGVH3-JH4 | Vg3-J2 | | | Vg5-Ig1.1/2.3F01<br>GTGGGGTTGTTAGGGGA | IGHV3-4F01<br>GAGCAGCTCGTCCTAAAA<br>CT |
| 25 | D2-JH3<br>D4-JH4 | | DD2-DD3 | DB1-J2-<br>7 | IGHV3-4F01<br>TGACTACGGTGACCTCGACTAC | |
| 26 | | Vg2-J2 | | DB1-J1-<br>1<br>DB2-J2-<br>5 | B2-5F01<br>CGGGTGGAGGAGCAAGA | Vg5-Ig1.1/2.3F02<br>TACTGTGCCACCTGT-<br>TAAGA |
| 27 | | Vg2-J2<br>Sil-Tal | | DB1-J1-<br>2 | Vg5-Ig1.1/2.3F02<br>ACAGGCTCCCCGCGA | TalF02 (189)<br>TGCATTCCT-<br>CACAATTCCCC |
| 28 | | | DD2-DD3<br>DD2-JD1 | | DD2-5DD3F01<br>TTTCATTGTGCCTTCCTACGA | JD1F01<br>TGTTTCATTGT-<br>GCCTTCCTAC |
| 28 | 20 | 16 | 17 | 6 | | |
| Total<br>(%) | 71.43 | 57.14 | 60.71 | 21.43 | | |

ately determined in order to be able to detect below $10^{-5}$.

From a total of 28 ALL patients diagnosed by MRD, 71.42% were found to have a rearrangement of immunoglobulins; 89.29% have a rearrangement of gamma T cell receptors; 64.28% have a rearrangement of delta T cell receptors; 21.42% have a rearrangement of beta T cell receptors. Of these 3 patients (10,71%) had 1 rearrangement, 4 patients (14,29%) had 2 rearrangements, 9 patients (32,14%) had 3 rearrangements, 10 patients

(35,72%) had 4 rearrangements, 2 patients (7,14%) had 5 rearrangements (*Table 2*).

All leukemikas can be regarded as a clonal expansion of initial leukemic cells. These clones can be characterized by junctional rearrangements of the immuno genes, which are identifiable during the screening procedure. After sequencing of the PCR products the junctions could be identified with the help of online tools like BLAST or IMGT and respective elements can be assigned. Nearly every junction of rearranged V- D- and J-elements results in a unique sequence providing the opportunity to design patient specific primers, which are complementary to this unique part. Within this study we designed for every junction two distinguishable oligonucleotides and performed functional testing with serial dilutions of leukemia blast DNA in DNA of healthy volunteers. Each primer was characterized with respect to detection limit and quantitative range. Frequently the unique sequences are suboptimal for designing PCR primers, since the purin pyrimidine ratio is unbalanced or sometimes nucleotides are clustered. Therefore we established simple rules for primer design. As a first attempt, the primer should cover only one base of downstream located germline element, while the second version should cover several nucleotides of this element. Out of 46 primer tested, 56.52% of the first (short) version were regarded as the better ones and in 41.30% the second version was superior.

We intended to establish two PCR amplicons targeting unrelated rearrangements per patient. In cases with more than two identified junctions we tested all per patient possibilities and finally selected the most appropriate ones. In cases where only one junction could be identified, only one marker was used.

Following this strategy for 18 of 28 patients a two marker approach could be realized (64.28%), and a one marker approach was restricted to 10 patients (35.71%). Observation period (as defined as the last available sample) was between 30 and 720 days post-transplant. During this period 67.85% of all patients were MRD negative. Of all samples which were determined MRD positive, 2 patients (7.14%) were positive below quantitative range, 1 patient (3.57%) between 0.01 % and 0.1 %, and 6 patients (21.42%) higher than 0.1 %.

We had eight patients with negative MRD and during observation the MRD became positive. Those patients (no 2 and 22) which were determined postive MRD (0.0001 %) in the early phase (D30 and D60) showed subsequent increase of MRD load, while those who were determined positive in the later phase (1 and 3 at day 400) did not. The latter might be due to the end of the observation period or might show interference with the immune reconstitution resulted in wrong positivity, as described by Fronkova et al. (31) (*Table 3*).

We had two patients (2 and 23) with strong positive MRD and during observation the MRD turned to negative due to clinical intervention. However, for 5 patients with high MRD load the induction of remission was not obtained.

## Conclusions

Real time PCR based detection of minimal residual disease in bone marrow distinguishes patients with respect to molecular remission after allogeneic transplantation. Additionally, it can enable detecting dynamic changes of MRD load during the observation course. As a perspective, a well characterized and homogenous patient cohort should be enrolled in a clinical study to investigate the impact of post-transplant MRD on the outcome.

**Aknowledgments**

The study was performed in the frame of POSDRU/88/1.5/S/63117 project, and at the Children's Hospital of the J.W. Goethe University, Frankfurt, Main, Germany.

**Conflict of interest declaration**

Authors declare no conflict of interests.

**Table 3. Results of MRD mesurement after transplantation**

| No. | D30 | D60 | D100 | D200 | D300 | D400 | D500 |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 1,00E -06 | 1,00 E-06 |
| 2 | 0 | 1,00E-06 | 1,30E-01 | 1,10E-01 | 1,00E-06 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 1,00 E-06 | 1,00 E-06 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 2,60E-01 | 7,00E-01 (recidive) | - | - | - |
| 15 | 0 | 0 | 0 | 0 | 0 | 2,50E-01 | 1,60E-02 |
| 16 | 0 | 1,00E-03 | 5,00E-04 | 0,00E+00 | 1,50E-04 | 3,00E-03 | - |
| 17 | 0 | 0 | 0 | 0 | 0 | - | - |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 1,70E-03 | 1,80E-01 | - | - | - |
| 21 | 0 | 0 | 0 | 0 | - | - | - |
| 22 | 1,00E-06 | 1,00E-06 | 1,00E-06 | 1,70E-02 | 1,70E-03 | 1,10E-02 | - |
| 23 | 1,10E-02 | 2,00E-03 | 2,60E-02 | 4,00E-03 | 1,00E-06 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 26 | 0 | 0 | 0 | 0 | 0 | - | - |
| 27 | 0 | 0 | 0 | 0 | - | - | - |
| 28 | 0 | 0 | 0 | 0 | - | - | - |

e.g. 1.10E-02 represents 1.1 leukemia cell beneath 100 or 1.1 %; 1.70E-03 represents 1.7 leukemia cells beneath 1,000 or 0.17 %

## Abbreviations list

ALL – Acute Lymphoblastic Leukemia
Real Time PCR – Real-time Polymerase Chain Reaction
MRD – Minimal Residual Disease
alloHSCT – Allogeneic Hematopoietic Stem Cell Transplantation
DNA – Deoxyribonucleic acid
RNA – Ribonucleic acid
ASO – Allele Specific Oligos
EDTA – EthyleneDiamineTetraacetic Acid
$IgVH_{1-6}$-$JH_{1-6}$ – Immunoglobulin Heavy chains
$IgV\kappa_{I-IV}$-$Jk_{1-5}$/ -k-deleting element – Immunoglobulin kappa light chain rearrangemnets
$TCRV\gamma_{I-IV}$-Jγ – gamma T cell receptors
$TCRV\delta_{1-3}$-$J\delta_1$; $V\delta_2$-$D\delta_3$; $D\delta_2$-$D\delta_3$; $D\delta_2$-$J\delta_1$ – delta T cell receptors
$TCRV\beta_{2-24}$–$J\beta_{1-2}$, $D\beta_{1-2}$-$J\beta_{1-2}$ – beta T cell receptors
Variable – V, diversity – D, joining – J elements of the immune receptor genes

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