

Univerzita Karlova v Praze

2. lékařská fakulta

Studijní program: Molekulární a buněčná biologie, genetika a virologie



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Next Generation Sequencing-based Analysis of Immunoglobulin and T-cell
Receptor Genes Rearrangements Repertoire in Haematological
Malignancies

Analýza repertoáru přestaveb genů pro imunoglobuliny a T-buněčné
receptory u hematologických malignit pomocí sekvenování nové generace

Disertační práce

Vedoucí závěrečné práce/Školitel: MUDr. Eva Froňková, Ph. D.

Praha, 2016

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Poděkování:

Děkuji především své školitelce MUDr. Evě Froňkové, Ph. D. za její pomoc, seznámení s tématem přestaveb antigenních receptorů, odborné vedení a dobré nápady.

Prof. MUDr. Janu Trkovi, Ph. D. bych ráda poděkovala za možnost pracovat v laboratorním centru CLIP a za odbornou pomoc v průběhu celého mého postgraduálního studia.

Prof. MUDr. Janu Starému, DrSc. děkuji za možnost pracovat na Klinice dětské hematologie a onkologie 2. LF UK a FN Motol.

Kolegům z laboratorního centra děkuji za pomoc, cenné rady a připomínky a za vytvoření příjemného pracovního prostředí.

Své rodině a přátelům děkuji za podporu a trpělivost, nejen v průběhu mého postgraduálního studia.

Identifikační záznam

KOTROVÁ, Michaela. *Analýza repertoáru přestaveb genů pro imunoglobuliny a T-buněčné receptory u hematologických malignit pomocí sekvenování nové generace. [Next Generation Sequencing-based Analysis of Immunoglobulin and T-cell Receptor Genes Rearrangements Repertoire in Haematological Malignancies]*. Praha, 2016. 72 s.,5 příl. Disertační práce (Ph. D.). Univerzita Karlova v Praze, 2. lékařská fakulta, Klinika dětské hematologie a onkologie. Vedoucí práce MUDr. Eva Froňková, Ph. D.

Abstract

We established next generation sequencing (NGS)-based assays for the detection of rearranged immunoglobulin (Ig) and T-cell receptor (TR) genes (Ig heavy chain complete/incomplete, TR gamma and delta). We used these assays for minimal residual disease (MRD) detection and antigen repertoire analysis in children with acute lymphoblastic leukaemia (ALL).

We compared MRD levels in 210 paediatric ALL samples detected by NGS and by quantitative PCR (qPCR), which is a current gold standard for MRD detection. We showed that both methods provide highly comparable results and that NGS results are more specific for relapse prediction. The better specificity of NGS was also proven on 40 post-transplant samples, where qPCR can provide false positive MRD results. We showed that repertoire diversity at day 78 is an independent prognostic factor in paediatric ALL.

Besides the above-mentioned advantages NGS-based MRD detection is cheaper and faster than qPCR. Before NGS-based MRD monitoring can safely replace qPCR, the extensive standardization of the method must be performed, as well as the evaluation of its prognostic relevance on a larger group of patients.

Keywords: Minimal residual disease, Next Generation Sequencing, Ig/TR rearrangements

Abstrakt

Zavedli jsme metodu pro sekvenování přestavěných genů pro imunoglobuliny (Ig) a T-buněčné receptory (TR) (kompletní/inkompletní přestavby těžkých řetězců Ig, TR gamma a delta), založenou na sekvenování nové generace (NGS), kterou jsme použili pro detekci minimální reziduální nemoci (MRD) a k analýze antigenního repertoáru u dětí s akutní lymfoblastickou leukémií (ALL).

Srovnali jsme hladiny MRD ve 210 vzorcích dětí s ALL detekované pomocí NGS a kvantitativní PCR (qPCR), což je v současnosti používaná standardní metoda pro detekci MRD. Ukázali jsme, že obě metody poskytují vysoce srovnatelné výsledky a že výsledky získané pomocí NGS jsou specifitější pro predikci relapsu. Lepší specifitu NGS jsme prokázali také na 40 post-transplantačních vzorcích, u kterých může docházet k detekci falešně pozitivních qPCR výsledků. Dále jsme ukázali, že diverzita antigenního repertoáru ve dni 78 je nezávislým prognostickým faktorem u dětských ALL.

Kromě výše zmíněných výhod je detekce MRD založená na NGS levnější a rychlejší než qPCR. Než však bude možno detekci MRD pomocí NGS nahradit qPCR, bude nutné provést rozsáhlou standardizaci této metody a také zhodnotit její prognostický význam na rozsáhlejší souboru pacientů.

Klíčová slova: Minimální reziduální nemoc, sekvenování nové generace, Ig/TR přestavby

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1. Introduction

1.1 Immunoglobulins and T-cell receptors

The human immune system protects the body against millions of various antigens. The ability of the immune system to recognize such a large number of antigens depends on B-lymphocytes and T-lymphocytes. Lymphocytes are a subtype of white blood cells, producing highly variable antigen receptors, which enable specific molecular recognition of antigens. T-cell receptors (TR) are present on the surface of T-lymphocytes and B-lymphocytes produce secreted or surface-bound immunoglobulins (Ig).

All types of immune receptors (Ig, TR) are composed of constant and variable domains¹. The antigen receptors' ability to recognize millions of different antigens is based on the great diversity of their variable domains². If the entire immune repertoire was encoded by individual genes, a large portion of the human genome would be occupied only by these genes. Instead, genes for variable regions are formed by rearrangements of a limited number of germline gene segments. This process is called somatic recombination and produces millions of different combinations of V, (D), and J segments³. Each lymphocyte bears many copies of the antigen receptor with a unique variable region which determines its antigen specificity.

Both surface-bound and secreted immunoglobulins consist of two heavy chains (IgH) and two light chains (IgL), which are connected by a disulphide bond. Genes for IgH chains are localized in a gene complex on chromosome 14q32.3. This gene complex consists of 46-52 (depending on the individual haplotype) V ("variable") segments at 5' end, which can be classified into 7 families based on their sequence homology. V segments are followed by a group of 27 D ("diversity") segments which can be grouped into 7 families and 6 short J ("joining") segments. The gene segments for constant (C) part of the heavy chain are localized at the 3'-end of the gene complex and are responsible for the immunoglobulin class determination⁴.

IgL chains are either encoded by a kappa complex (IgK) which is localized on chromosome 2p11.2, or by a lambda complex on chromosome 22. The structure

of these complexes resembles the IgH complex, but it is composed of less V and J segments and does not contain D segments.

TR molecules are also composed of two chains, connected by a disulphide bond. The „classical“ type of TR contains α (TRA) and β (TRB) chains, whereas the „alternative“ type contains γ (TRG) and δ (TRD) chains^{5,6}. The variable domains of TRB and TRD contain all three types of gene segments (V, D and J). TRA and TRG chains lack D segments similarly to immunoglobulin light chains. The gene complex for TRD is localized within the gene complex for TRA (between the V and J segments). Any rearrangement of the TRA gene segments thus results in the loss of TRD gene segments, which means that rearranged TRA and TRD loci can never be present together in a single cell.

1.1.1 Somatic recombination

The somatic recombination (Figure 1) occurs in immature lymphocytes in primary lymphoid organs (bone marrow for B-lymphocytes, thymus for T-lymphocytes). V, D and J segments are flanked by so called RSSs (recombination signal sequences), which are recognized by a group of proteins responsible for the V-(D)-J recombination. During this recombination V, D and J gene segments of Ig and TR genes are rearranged and a DNA sequence, which is unique for each lymphocyte, is produced.

V(D)J recombination of IgH and TRB genes is a two-step process including D-J recombination followed by V-D recombination. The recombination of the TRD locus starts with D-D recombination (up to 3 D segments can form the final coding joint) and continues with V-D and D-J recombinations. Genes for IgK, IgL, TRA and TRG are produced by a one-step V-J recombination. During each recombination random number of gene segments is excised and the remaining segments are joined together. This gives rise to tens of millions of possible combinations of V, (D) and J segments.

This process occurs in parallel on both chromosomes. As soon as the productive rearrangement is formed, it is transcribed into mRNA. At that point the recombination

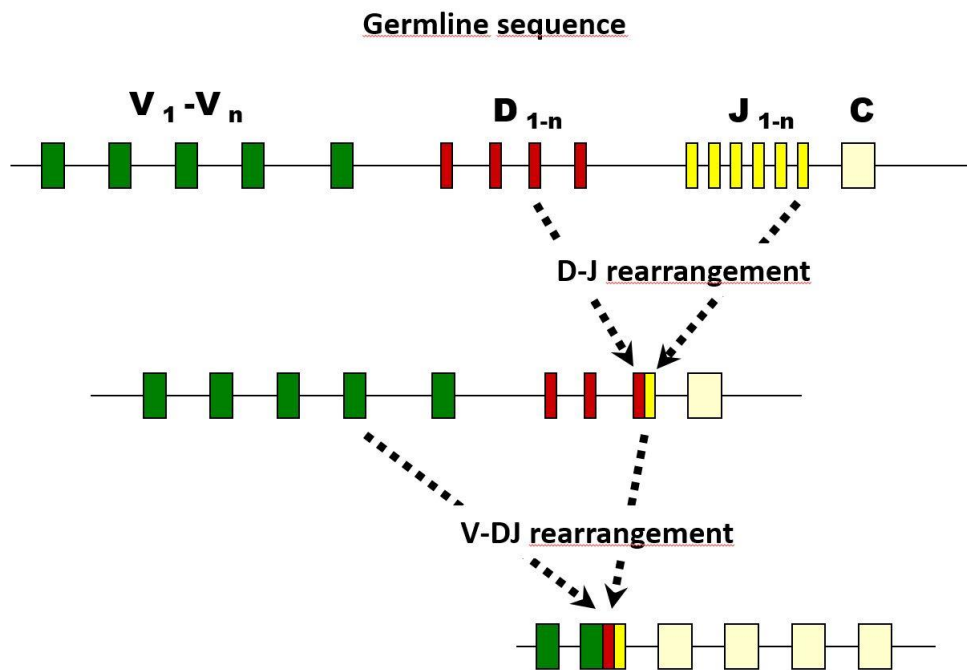


Figure 1: The scheme of somatic recombinations.

process on the second chromosome is stopped and therefore only one type of antigen receptor is produced by each lymphocyte (allelic exclusion^{7,8}).

1.1.2 Junctional diversity

The joining of V, D and J gene segments is a very imprecise procedure: the ends of germline segments which are being joined are cleaved randomly. This further increases the diversity of immune repertoire³.

Moreover the enzyme called terminal deoxynucleotidyl transferase randomly adds up to 20 non-templated nucleotides (N-bases) at the junctions between the gene segments which are joined together. This further increases the variability of these sequences.

As a result of the above-mentioned processes antigen receptors (which are encoded by rearranged Ig or TR genes) are highly diverse and are able to recognize virtually all possible antigens.

1.1.3 Affinity maturation

Moreover, after the recognition of antigen by B-lymphocytes in secondary lymphoid organs (lymph nodes) an enzyme called activation-induced cytidine deaminase introduces somatic hypermutations around the productively rearranged V(D)J junction⁹ and cells with higher affinity for the antigen are favoured^{10,11}. This event, called affinity maturation, enables production of immune receptors with very high affinity to the certain antigen¹².

1.1.4 Antigen repertoire and clones

The entire set of antigen receptors with different antigen specificities in one individual is called the immune repertoire. In humans it is believed to be 10^{11} or higher¹.

Thanks to the recombination process each newly developed B- or T-lymphocyte carries a unique sequence coding its antigen receptors. The entire clone of cells that arises by divisions of this newly developed lymphocyte carries the same sequence as the “mother” lymphocyte.

Lymphoid malignancies are clonal diseases resulting from the malignant transformation of a single lymphocyte and its subsequent expansion, and therefore the entire malignant clone carries identical Ig/TR V(D)J rearrangement(s), which can be used for molecular detection of malignant cells.

1.2 Next generation sequencing

Next generation sequencing (NGS) is a revolutionary technology producing up to billion unique sequences (“reads”) within one sequencing run. The high throughput of next generation sequencers is commonly used for sequencing of the whole genomes, exomes or transcriptomes of different organisms, i.e. for covering huge genomic regions. On the other hand NGS can also be used for sequencing of targeted small genomic areas with ultra-high coverage. This application of NGS can be also used for detection of MRD.

The biggest advantage of next generation sequencing over conventional Sanger sequencing is the ability to produce an enormous volume of data fast and cheaply. This is enabled by sequencing of millions short DNA fragments in parallel. To achieve this, the starting DNA must be processed before sequencing in two steps consisting of the library preparation and template preparation.

1.2.1 Library preparation

The first step of library preparation is the random fragmentation of starting DNA using physical or chemical methods¹³. Adaptors specific for each sequencing platform and sample-specific barcodes, which enable sequencing of many samples within one sequencing experiment, are then ligated to the DNA fragments¹⁴. After adaptor ligation, DNA fragments are amplified using primers complementary to the adaptor sequences.

Libraries can also be prepared by PCR amplification using so called “fusion primers”. These primers contain a sequence specific for the amplified region and also sequencing adaptors specific for the sequencing platform (and can also contain barcodes) at their 5'-ends. After amplification, PCR products (amplicon libraries) are flanked by sequencing adaptors on both sides and can also be flanked by sample-specific barcode(s) either on one or both ends.

1.2.2 Template preparation

Following library preparation, the adaptor-flanked fragments are quality-checked and quantified. Because conventional sequencers are unable to detect a signal from a single sequencing reaction, the amplification of sequencing template is needed: library fragments are either attached to micron-scale beads and amplified via emulsion PCR, or attached to a solid surface and amplified via bridge amplification¹⁵. These processes lead to the formation of clonally clustered amplicons: PCR products derived from a single library molecule are either spatially clustered on a solid surface or on micron-scale beads and serve as sequencing templates¹⁴.

1.2.3 Sequencing

Millions of sequencing reactions are performed in parallel, and therefore NGS technology is often called massively parallel sequencing (MPS). Currently there are two sequencing technologies dominating the market: Ion Torrent (PGM, Proton and S5 sequencers) and Illumina (MiSeq, HiSeq and NextSeq sequencers) technologies, both employing sequencing-by-synthesis approach.

Ion Torrent employs so called semiconductor technology¹⁶ – hydrogen protons are released as nucleotides are incorporated during DNA synthesis. This causes a change of pH which is detected as a voltage change by the semiconductor layer of the sequencing chip.

Illumina sequencers, which are currently most widely used¹⁷, utilize fluorescently labelled reversible-terminated nucleotides¹⁶. Only one nucleotide can be incorporated into a newly synthesized strand at a time and the fluorescence is detected after the incorporation.

1.3 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) is a malignancy which affects early progenitors of lymphocytes in the bone marrow. It is characterized by an increased proliferation of immature malignant white blood cells called lymphoblasts. The production of lymphoblasts takes place in the bone marrow, where it inhibits the production of normal blood cells (erythrocytes, lymphocytes and thrombocytes) and leads to anaemia, neutropenia, and thrombocytopenia.

Paediatric ALL is the most common childhood malignancy which represents approximately 25% of cancers diagnosed among children under 15 years. The peak of the incidence of ALL is between 2 and 5 years of age. Survival rates for childhood ALL have improved significantly during last decades¹⁸ and are currently reaching 90%¹⁹ thanks to the risk-adapted therapy and improved supportive care. However relapses still occur in 20% of children²⁰ with ALL and are associated with a poor outcome²¹.

1.3.1 Genomic characterization of childhood ALL

The majority (approximately 75%) of paediatric ALL cases carry chromosomal aberrations detectable by conventional cytogenetics²². In B-precursor ALL these include: high hyperdiploidy (non-random gain of at least 5 chromosomes), hypodiploidy (fewer than 44 chromosomes), and chromosomal translocations including t(12;21) encoding ETV6-RUNX1 fusion (also known as TEL-AML1) which is present in about 25% of cases, TCF3-PBX1 fusion (also known as E2A-PBX) encoded by t(1;19), t(9;22) encoding BCR-ABL1 fusion gene resulting in constitutive tyrosine kinase activity, rearrangements of MLL gene at chromosome 11q23 with many different translocation partners, P2RY8-CRLF2 translocation and rearrangements of IgH with many translocation partners (CRLF2, BCL2, EPOR, ID4, IL3, CEBPE). High hyperdiploidy and ETV6-RUNX1 are associated with favourable prognosis and, on the other hand, MLL rearrangements, BCR-ABL1 and low hypodiploidy are associated with a poor outcome.

1.3.2 Submicroscopic genetic alterations

High resolution genomic methods (microarray profiling and next generation sequencing) have been under extensive development in the last years and have led to identification of novel structural genetic alterations and sequence mutations. Genes most commonly affected by mutations belong to regulators of lymphoid development (PAX5, IKZF1, and EBF1), regulators of cell cycle, tumour suppressors, are involved in RAS signalling (NRAS, KRAS and NF1) and cytokine receptor signalling (IL7R, JAK2) pathways.

1.3.3 Risk assignment

Age at diagnosis has a strong prognostic impact. Children between 1 and 9 years of age have better outcome than infants and adolescents/young adults²³. Older children have a greater prevalence of biologically high-risk leukaemia. Presenting

leukocyte count is another prognostic variable – increasing counts are associated with a poorer outcome. The poorer prognosis associated with black²⁴ and Hispanic²⁵ ethnicity and male sex have been also proven in some studies.

Response to treatment is also predictive of the relapse risk and recent studies have shown that its prognostic power is even greater than that of the “classical” prognostic markers. It is currently used for the stratification of patients into the risk groups and administration of risk-adapted therapy. This topic is discussed in the next section.

1.4 Minimal residual disease

The term minimal residual disease (MRD) is used to describe the low-level disease which is not detectable by cytomorphology (which can reach the sensitivity of 10^{-2}) and is not accompanied by clinical symptoms. It can be detected only by sensitive techniques like quantitative polymerase chain reaction (qPCR) or flow cytometry (FC)²⁶. The MRD decrease after the administration of remission induction therapy reflects the effectiveness of such treatment in a particular patient.

1.4.1 Clinical significance of MRD

MRD is currently the most important prognostic factor in childhood acute lymphoblastic leukaemia²⁷, independent of other relevant risk factors such as age, sex, immunophenotype, presence of chromosome aberrations at diagnosis, prednisone response and white-blood-cell count.

It was previously shown that MRD detection at single time point is not sufficient for defining the risk of relapse²⁸ and therefore MRD at two time points is currently used for MRD-based risk stratification on frontline treatment protocols: MRD at the end of induction therapy (5th week of treatment) is useful for recognition of patients with low risk of relapse and MRD at the end of consolidation therapy (12th week of treatment) for recognition of patients at high risk.

Currently MRD is routinely used for guiding clinical decisions in children with ALL: based on MRD levels patients are stratified into four risk groups, which differ in the intensity of chemotherapy^{29,30}. Patients are considered MRD standard risk (MRD-SR) if MRD analysed with a sensitivity of at least 10^{-4} is negative at both day 33 and day 78, MRD high risk (MRD-HR) if MRD level is 10^{-3} or higher at day 78, MRD slow early responders (SER)³¹ if MRD at day 33 is above 10^{-3} and positive at day 78. The remaining patients are considered MRD intermediate risk (MRD-IR).

Patients with bad response to initial therapy are assigned into the high risk treatment arm and receive intensified chemotherapy. On the other hand, patients with prompt MRD response are classified as low risk and undergo randomization aiming at de-escalation of therapy with reduced toxicity and fewer long-term side effects.

MRD can also be used to predict outcome and choose the optimal therapy in children with relapsed ALL: it was shown that an early MRD response after chemotherapy administration is associated with good prognosis and transplantation is not necessary in children with prompt MRD response³². Strong predictive value of MRD before transplantation on outcome after transplantation was also shown³³. Children, who are MRD-positive before transplantation, have extremely poor prognosis³⁴.

1.4.2 MRD detection

MRD is usually expressed as the proportion of leukemic cells from total number of tested bone marrow cells (e. g. one leukemic cell in 1000 cells is expressed as MRD load of 10^{-3}).

Leukemic cells have unique genetic and immunophenotypic signatures. Three techniques are currently used to detect MRD with sufficiently high sensitivity:

- 1) Flow cytometric detection of aberrant immunophenotypes;
- 2) PCR detection of breakpoint fusions of leukaemia-specific chromosome aberrations (e. g. BCR-ABL, MLL-AF1, TCF3-PBX and ETV6-RUNX1);

3) PCR detection of leukaemia-specific antigen receptors genes rearrangements.

These three methods can reach the sensitivity of 10^{-3} to 10^{-5} ³⁵.

As mentioned above, MRD is usually monitored in bone marrow samples. Monitoring of MRD in peripheral blood would avoid bone marrow aspirations which are very traumatic for children. But MRD levels in peripheral blood were shown to be on average 11.7-fold lower than in bone marrow³⁶ and therefore theoretically ten-fold higher sensitivity of MRD techniques would be needed for monitoring of MRD in peripheral blood.

1.4.2.1 qPCR-based detection of rearranged Ig and TR genes

Quantitative PCR-based detection of rearranged junctional regions of Ig and TR genes is a laborious and time-consuming process involving many steps. In order to obtain comparable results between different laboratories strict guidelines for experimental approaches and data interpretation were developed during multiple rounds of international quality controls^{37,38}. The extensive experience with Ig/TR genes rearrangements is also necessary.

The sequence of leukaemia-specific Ig and TR gene rearrangements can be identified at diagnosis via PCR amplification with universal primers and a subsequent Sanger sequencing of the resulting PCR products. Such targets can be identified in more than 95% of patients with lymphoid malignancy³⁹.

The obtained sequence of the V-(D)-J junction is further used to design clone-specific probes or primers, also known as allele-specific oligonucleotide (ASO) probes and primers. The leukemic clone carrying the sequence identified at diagnosis can then be quantified by qPCR either employing clone-specific junctional region probes or employing clone-specific junctional region primers⁴⁰⁻⁴⁴.

In a routine practice the optimization of such assay takes approximately 4 weeks: After the separation of mononuclear cells, DNA extraction and PCR screening, the PCR products are sequenced in the sequencing core facility, which usually takes at least one week. When the sequences of the targets are obtained, the target-specific

primers are designed and ordered. The primer synthesis also takes at least one week. After the primers are delivered, the optimal PCR conditions for the assay must be found and subsequently used for the detection of MRD in follow-up samples.

It is important to monitor ALL patients with at least two PCR targets, because ongoing rearrangements and secondary rearrangements accompanying the disease can cause the change or loss of the junctional sequence from diagnosis and consequently the false negative results²⁸.

1.4.2.2 Flow cytometric detection of MRD

Flow cytometric MRD detection is faster, less expensive and is applicable in higher percentage of patients than qPCR-based detection. This is the reason why FC is used for identification of very early responders (<0.1% blast cells at day 15) in the current treatment protocols, who may benefit from treatment reduction, and also patients with high MRD levels ($\geq 10\%$ blast cells at day 15), who may benefit from treatment intensification^{45,46}.

1.4.2.3 Next generation sequencing MRD detection

For amplicon NGS-based MRD detection no patient-specific primers are needed. Libraries are prepared using consensus primers complementary to the terminal parts of junctional regions which are not as diverse as their central parts. These primers should amplify virtually all rearranged Ig and/or TR genes present in the sample. Because same primers are used for all patients, no optimization of patient-specific assays is needed, as is for qPCR.

This method provides the information about the sequence and abundance of each clone in the tested sample and enables its tracking also in follow-up samples. Because the sequences of the entire CDR3 regions (allowing for specific identification of leukaemia-specific clones) are provided, false negative MRD results caused by clonal evolution during the disease course and also false positive results caused by low specificity of qPCR primers⁴⁷ should be avoided.

NGS is expected to reach the sensitivity of 1 cell in 10^6 cells, assuming that a sufficient number of cells is analysed. However, this is usually impossible in the situation of severe bone marrow aplasia. This sensitivity is ~10 times higher than the sensitivity which is usually reached with qPCR. It could be theoretically used for MRD quantification in peripheral blood, where the MRD levels are usually an order of magnitude lower than in bone marrow⁴⁸.

One of the first studies describing MRD detection by amplicon NGS using 454 pyrosequencing was published in 2011⁴⁹. It was performed on post-transplant chronic lymphocytic leukaemia (CLL) samples, using BIOMED-2 consensus primers⁵⁰, and observed correlation between MRD detected by qPCR and NGS was high.

The prognostic relevance of post-transplant IgH-based NGS-MRD in CLL was confirmed two years later by a commercial laboratory (Sequentia)⁵¹, applying a LymphoSIGHT method⁵².

LymphoSIGHT method

LymphoSIGHT is a high-throughput sequencing-based commercial method, employing consensus primers for IgH (complete and incomplete), IgK, TRG, TRB and TRD rearrangements to amplify all rearranged Ig and TR genes in the sample⁵². The known copy numbers of reference IgH sequences are added into each sample and the numbers of reads covering the reference sequences are then used for calculating the coverage of each molecule. This information is then used to obtain the absolute number of leukaemia-associated molecules in the sample. The authors claim that the LymphoSIGHT method is quantitative at frequencies above 10^{-5} and that the sensitivity of this method is below 10^{-6} . However they also admit that the sensitivity is limited by the number of analysed cells, which can be a real limitation in aplastic samples from patients on treatment. The technical performance of the LymphoSIGHT platform was tested on 106 follow-up paediatric ALL samples. MRD results obtained by NGS, FC and qPCR were then compared: the high degree of concordance was demonstrated among all the three methods.

In 2014 the comparison of LymphoSIGHT IgH-based NGS MRD detection with qPCR-based MRD detection was demonstrated on 55 patients with ALL/mantle cell lymphoma (MCL)/multiple myeloma (MM)⁵³. It was proven that the NGS-based identification of clonotypes is at least comparable to qPCR. Somatic hypermutations of the V-segments in MM were identified as the cause of failure in both NGS and qPCR. Although some discordant MRD results were obtained by the two methods, the final correlation was high.

Logan et al. used the LymphoSIGHT method for MRD detection in adult B-ALL patients before and after transplantation⁴⁸. Employing all the targets (IgH complete/incomplete, IgK, TRB, TRD, TRG) it was possible to detect MRD in 93% of all patients. MRD $> 10^{-4}$ before transplantation and MRD $> 10^{-6}$ after transplantation were shown to be associated with the subsequent relapse.

ImmunoSEQ (ClonoSEQ) method

In 2012 Wu et al. used Illumina HiSeq platform and another commercially available method called clonoSEQ⁵⁴ (developed by Adaptive Biotechnologies) for sequencing of the variable regions of TRB and TRG genes and used the assay for clonality testing at diagnosis and for MRD detection at later time points in T-lineage ALL/lymphoma patients⁵⁵. MRD levels detected by NGS were then compared with results from multiparametric FC. As expected, it was shown that the detection threshold of NGS is lower than the detection threshold of FC. MRD detection based on amplicon NGS of TRB rearranged genes was shown to be more specific than MRD detection based on TRG amplicon NGS due to greater germline diversity of the TRB locus.

Two years later Wu et al. published the data regarding amplicon NGS of IgH locus in unselected paediatric B-ALL samples, again in cooperation with Adaptive Biotechnologies⁵⁶. In 91 of 98 (93%) sequenced patients the identification of IgH leukaemia-specific clonal rearrangement (representing more than 10% of all rearrangements) in the diagnostic samples was successful. Concordantly with their previous work it was shown again that NGS is more specific than FC. Interestingly this

cohort contained five samples, which were negative by FC and NGS detected MRD at levels higher than 10^{-4} , which should have been detected also by FC. It was demonstrated that these patients' leukaemia-specific sequences were present in the clones which were immunophenotypically mature. These were probably the initial leukemic clones which underwent antigenic maturation on therapy and thus could not be detected by FC.

Pulsipher et al. used the ImmunoSEQ assay for MRD detection in pre- and post-transplant samples from paediatric B-ALL patients and compared the relapse-prediction based on MRD detected by NGS and FC⁵⁷. It was not surprising that NGS which is supposed to be more sensitive predicted relapse more accurately than FC.

To provide precise and reliable MRD quantification Adaptive Biotechnologies developed an approach for correction of amplification bias of various V(D)J rearrangements in multiplex PCR⁵⁸. In such biased PCR product the representation of each rearrangement is not proportional to its frequency in the original sample thus making the accurate quantification of the leukemic clone impossible. For the purpose of amplification bias correction the synthetic repertoire with all possible V/J combinations was generated and used for adjusting primers concentrations in multiplex PCR. Residual differences in the amplification efficiency were then removed computationally.

Target screening

It was shown that also only replacement of classical Sanger sequencing-based target screening by NGS can be highly beneficial⁵⁹. Thirty-four paediatric patients were prospectively analysed during this study and it was demonstrated that more markers for each patient can be detected by NGS than by classical PCR screening combined with Sanger sequencing. Also the number of sequencing failures was reduced when using NGS. NGS-based method was faster and easier to use in a routine practice.

1.4.2.4 Ongoing clonal evolution in ALL

It has been previously shown that ALL cells often undergo extensive changes of Ig and/or TR rearrangements between diagnosis and relapse⁶⁰ mediated by ongoing activity of the V(D)J recombinase enzymes⁶¹. This phenomenon, also known as clonal evolution, may lead to false negative qPCR-MRD results.

Szczepanski et al.⁶² proved that the discrimination between monoclonality and oligoclonality at diagnosis strongly predicts the clonal evolution during the disease course: targets which were monoclonal at diagnosis were highly stable, whereas less than half of the diagnostic oligoclonal targets were preserved at relapse.

It was also demonstrated that at least one rearrangement remains stable at ALL relapse. It is therefore recommended to use junctional regions of at least two independent genes for MRD monitoring to avoid false negative results.

Two distinct mechanisms can cause changes in the antigen receptor genes loci:

- 1) V-replacement: original V segment is replaced by another V segment, but the D-J part of the rearrangement remains unaffected.
- 2) The incomplete leukemic D-J rearrangement may continue the recombination process and can recombine with more different V segments.

NGS amplicon sequencing of rearranged Ig/TR genes can detect new rearrangements resulting from clonal evolution and can also distinguish which of the above-mentioned mechanisms is responsible for their occurrence⁶³.

1.4.2.5 Advantages of in-house NGS-based MRD assay

We decided to develop our own NGS-based assay for MRD detection, despite the fact that two non-academic companies had already started to present their results. The substantial disadvantage of commercial assays is the fact that the detailed description of the methodology is lacking. Thus, there is no possibility to assess the reliability of the generated data. Moreover, the wrong interpretation of MRD results in a sample with B-cell aplasia resulting in a substantial overestimation of real MRD value appeared in a case report⁶⁴ employing one of the commercial approaches.

2. Aims

I. To design, develop and validate NGS-based assay for the detection of rearranged Ig and TR genes

- a. to design fusion primer sets for amplification of virtually all possible combinations of gene segments for IgH (both complete and incomplete), TRD and TRG rearrangements
- b. to optimize PCR-based library preparation using our in-house fusion primers
- c. to develop bioinformatic algorithm for data analysis

II. Use the developed assay for:

- a. the **screening of MRD targets** in the diagnostic DNA samples from patients with paediatric B-precursor ALL and mantle cell lymphoma (MCL), where the classical screening procedure employing PCR amplification with universal primers and Sanger sequencing failed, and use these targets for MRD detection using classical qPCR-based technique
- b. **MRD detection** in the DNA samples from the prognostically relevant time-points (days 15, days 33 and days 78 from the beginning of treatment) of paediatric B-precursor ALL patients and compare the MRD detected by NGS with the MRD detected by Ig/TR – based qPCR
- c. **sequencing of the entire immune repertoire** in paediatric B-precursor ALL patients' DNA samples from days 15, 33 and 78 and assessing the repertoire diversity in these samples
- d. MRD detection in the **samples from paediatric B-precursor ALL patients after hematopoietic stem cell transplantation (HSCT)**, which were low MRD-positive

by Ig/TR – based qPCR and were not followed by relapse despite the fact that no additional chemotherapy was administered. We expect that NGS will help us to decide whether these samples are really positive or whether these positivities are false, caused by the massive regeneration of B-cells and T-cells after the HSCT. Moreover, the NGS can **uncover the real source of these qPCR positivities**.

III. To assess the prognostic significance of NGS-MRD and the repertoire diversity in paediatric B-precursor ALL patients

3. Hypothesis

We hypothesized that NGS for the antigen repertoire will have a number of benefits compared to current standard qPCR:

- we assumed that NGS will be cheaper, less laborious and faster than classical qPCR-based technique
- the higher speed of NGS analysis would enable on-line MRD detection also at early time points during treatment (e.g. at day 15); this is not possible with currently used qPCR-based technique, because the establishment of patient-specific qPCR assays takes at least 4 weeks in the routine practice
- unlike qPCR, NGS can provide not only the information about the level of MRD, but also the information about the number and abundance of various clones in the sample, thus enabling us to monitor the immune reconstitution after chemotherapy or transplantation and evaluate its prognostic relevance for relapse prediction
- because the V(D)J junction sequences of the entire repertoire can be obtained by NGS, it will enable also the detection of subclones and biallelic rearrangements within one sequencing experiment
- assuming that MRD detection by NGS is more specific than classical qPCR-based technique (in which non-specific primer annealing can cause positive MRD results, even if the leukaemia-specific clone from the diagnosis is not present) we expect that MRD detected by amplicon NGS will provide more reliable prediction of relapse
- we believe that highly specific MRD detection by amplicon NGS will therefore confirm or disprove low positivities in post-transplant samples from patients who did not subsequently relapse despite no chemotherapy intensification was

administered; in case these positivities are false, NGS will reveal which clone causes the false positive qPCR result

- in ALL samples from patients with ongoing clonal evolution after malignant transformation NGS-MRD detection should be possible and reliable, but the qPCR-based MRD detection will fail to detect evolved leukemic clones

4. Methods

4.1 DNA isolation from mononuclear cells

Bone marrow samples from the above-mentioned time points were subjected to Ficoll-Paque density centrifugation (Pharmacia, Uppsala, Sweden), through which mononuclear cells can be separated from the other cell types in the sample.

DNA was isolated from the mononuclear cells using QIAamp DNA Mini/Micro Kits (Qiagen). The quality and quantity of the DNA was measured by the NanoDrop spectrophotometer.

4.2 Library preparation

Libraries for MRD detection and repertoire analysis were prepared from 450 ng (this is the equivalent of approximately 70 thousand cells) of patients' DNA from days 15, 33 and 78 from the beginning of treatment and 50 ng of Human Genomic DNA (Roche). We assume that 70 thousand cells are enough to reach the sensitivity comparable with qPCR (which is usually 10^{-4} or 10^{-5}). Human Genomic DNA isolated from the peripheral blood of healthy donors was added to correct the low amount of B-cells in the aplastic samples and to prevent overamplification of present rearrangements, which can cause the overestimation of MRD (unpublished observation).

Libraries for MRD detection and repertoire analysis in post-transplant samples were prepared from 500ng of patients' DNA. No Human Genomic DNA was added, because regenerating post-transplant samples provide adequate background of physiological IgH rearrangements.

Libraries for target identification were prepared from 50ng of the diagnostic DNA. This amount of DNA is sufficient for target screening, because the infiltration of bone marrow at diagnosis is usually above 50% and therefore there is no need for high sensitivity. Human Genomic DNA was also not added.

Two round PCR was used for library preparation (Figure 2). In the first PCR all possible V-J combinations (or D-J combinations in case of incomplete rearrangements)

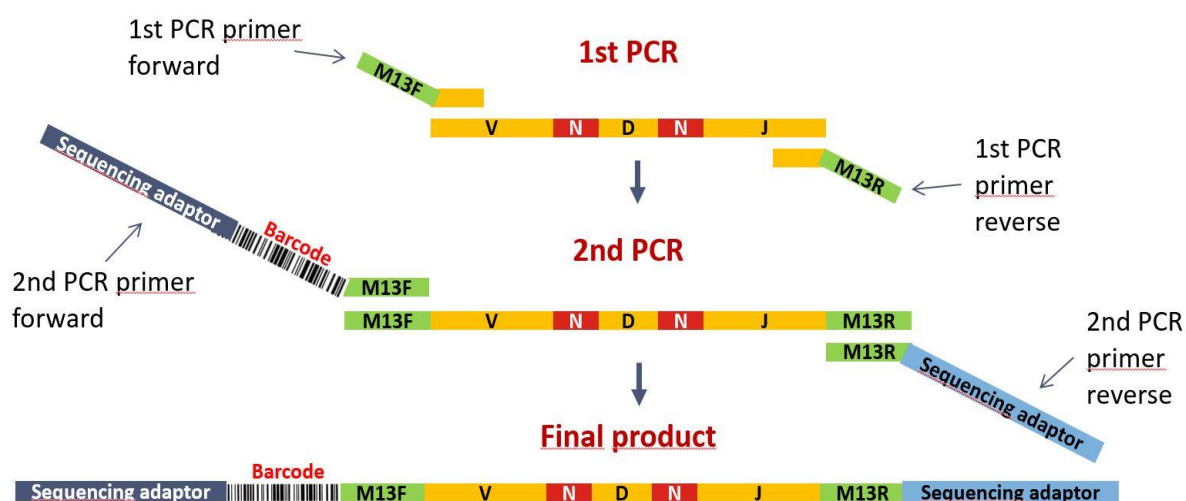


Figure 2: Library preparation via 2 round PCR.

were amplified using primer mixes designed for IgH (complete or incomplete), TRG or TRD targets. Primers for the first round (Table 1) also contained universal M13 adaptors at their 5'-ends, which were used in the second PCR for the amplification of 1st round products.

After the 1st PCR the products were purified by Agencourt AMPure XP beads (Beckman Coulter) using the beads:DNA ratio 1.5 (i. e. 10µl of the PCR product and 15µl of beads) and diluted 50 times prior to the 2nd PCR.

Primers for 2nd PCR (Table 2) contained M13 adaptors at 3'-ends and sequencing adaptors for the Ion Torrent sequencers with the sample-specific sequencing barcodes (enabling for sequencing of many samples within one sequencing run) at 5'-ends.

Each DNA molecule of the final library thus carried the sequence of the V(D)J rearrangement in the middle, which was flanked by sequencing adaptors and sequencing barcode. The PCR protocols are shown in Table 3.

System	Oligo Name	Oligo Sequence (5' to 3')
IgH (complete)	forward primer VH1	GTAAACGACGCCAGTGGAGCTGAGCAGCCTGAGATCTGA
	forward primer VH2	GTAAACGACGCCAGCAATGACCAACATGGACCCTGTGGA
	forward primer VH3	GTAAACGACGCCAGTCTGCAAATGAACAGCCTGAGAGCC
	forward primer VH4	GTAAACGACGCCAGGAGCTCTGTGACCGCCGCGGACACG
	forward primer VH5	GTAAACGACGCCAGCAGCACCGCCTACCTGCAGTGAGAGC
	forward primer VH6	GTAAACGACGCCAGGTTCTCCCTGCAGCTGAACCTCTGTG
	forward primer VH7	GTAAACGACGCCAGAGCCTAAAGGCTGAGGACAC
	reverse primer JH consensus	CAGGAAACAGCTATGACCTTACCTGAGGAGACGGTGACC
TRG	reverse primer Jg1Jg2	CAGGAAACAGCTATGACGTGTGTCCACTGCCAAAGAG
	reverse primer JgP1	CAGGAAACAGCTATGACAGGTGAAGTTACTATGAGCTTAGT
	reverse primer JgP2	CAGGAAACAGCTATGACAGGCGAAGTTACTATGAGCCTAGT
	reverse primer JgP	CAGGAAACAGCTATGACCGGGACCAAATACCTTGATTTT
	forward primer Vg1	GTAAACGACGCCAGAAATCTAATTAATAATGATTCTGGG
	forward primer Vg2	GTAAACGACGCCAGAAATCTAATTAATAATGACTCTGGG
	forward primer Vg35	GTAAACGACGCCAGAAATCTAATTAATAATGATTCTGGG
	forward primer Vg4	GTAAACGACGCCAGGAAATCTTATTGAAATGACTCTGGA
	forward primer Vg8	GTAAACGACGCCAGAAATCTAATTAATAATGACTCTGG
	forward primer Vg9	GTAAACGACGCCAGCAATGTAGAGAAACAGGACATAGCTA
	forward primer Vg10	GTAAACGACGCCAGCGTAGAGAAAGAAGACATGGCC
	forward primer Vg11	GTAAACGACGCCAGAAGTTCTTAGAGAAAGAAGATGAGGTG
IgH (incomplete)	forward primer DH1	GTAAACGACGCCAGAYCCAGGAGGCCCCAGAGCWCA
	forward primer DH2	GTAAACGACGCCAGCAGCACTGGGCTCAGAGTCCTCTC
	forward primer DH3	GTAAACGACGCCAGCCTCCTCMGGTCAGCCCYGGACAT
	forward primer DH4	GTAAACGACGCCAGCCCAGGACGACGACCRCTGTCAA
	forward primer DH5	GTAAACGACGCCAGTTCCTGGGAAGCTCCTCCTGAC
	forward primer DH6	GTAAACGACGCCAGCAGGCCCCCARAACCAGKGWT
	forward primer DH7	GTAAACGACGCCAGGCTAACAGCGGAAGGGAGAGCACT
	reverse primer JH consensus	CAGGAAACAGCTATGACCTTACCTGAGGAGACGGTGACC
TRD	forward primer Vd1	GTAAACGACGCCAGATGCAAAAAGTGGTCGCTATT
	forward primer Vd2	GTAAACGACGCCAGATACCGAGAAAAGGACATCTATG
	forward primer Vd3	GTAAACGACGCCAGGTACCGGATAAGGCCAGATTA
	forward primer Vd4	GTAAACGACGCCAGATGACCAGCAAAATGCAACAG
	forward primer Vd5	GTAAACGACGCCAGACCCTGCTGAAGGTCTACAT
	forward primer Vd6	GTAAACGACGCCAGCCCTGCATTATTGATAGCCAT
	forward primer Dd2	GTAAACGACGCCAGAGCGGGTGGTGATGGCAAAGT
	reverse primer Dd3	CAGGAAACAGCTATGACTGGGACCCAGGGTGAGGATAT
	reverse primer Jd1	CAGGAAACAGCTATGACGTTCCACAGTCACCGGGTTC
	reverse primer Jd2	CAGGAAACAGCTATGACGTTCCACGATGAGTTGTGTTT
	reverse primer Jd3	CAGGAAACAGCTATGACCTCACGGGGCTCCACGAAGAG
	reverse primer Jd4	CAGGAAACAGCTATGACTTGTACCTCCAGATAGGTTCC
	reverse primer Ja29	CAGGAAACAGCTATGACGGCAAAAGCATTCTAGGTACA

Table 1: Primers for 1st PCR. The primers for IgH (complete and incomplete) and TRD were adapted from van Dongen et al., Leukemia 2003. The primers for TRG were newly designed. Red: universal adaptor; black: sequence-specific primer.

Oligo Name	Oligo Sequence (5' to 3')
reverse primer	CCTCTCTATGGGCAGTCGGTGATCAGGAAACAGCTATGAC
forward primer with barcode 1	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACGATGTAAAACGACGGCCAG
forward primer with barcode 2	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACGATGTAAAACGACGGCCAG
forward primer with barcode 3	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCGATGTAAAACGACGGCCAG
forward primer with barcode 4	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGATGTAAAACGACGGCCAG
forward primer with barcode 5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGATGTAAAACGACGGCCAG
forward primer with barcode 6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCGATGTAAAACGACGGCCAG
forward primer with barcode 7	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCGATGTAAAACGACGGCCAG
forward primer with barcode 8	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACGATGTAAAACGACGGCCAG
forward primer with barcode 9	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACGATGTAAAACGACGGCCAG
forward primer with barcode 10	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACGATGTAAAACGACGGCCAG
forward primer with barcode 11	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTCGAATCGATGTAAAACGACGGCCAG
forward primer with barcode 12	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCGATGTAAAACGACGGCCAG
forward primer with barcode 13	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACGATGTAAAACGACGGCCAG
forward primer with barcode 14	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTTCGATGTAAAACGACGGCCAG
forward primer with barcode 15	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCGATGTAAAACGACGGCCAG
forward primer with barcode 16	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACGATGTAAAACGACGGCCAG
forward primer with barcode 17	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCGATGTAAAACGACGGCCAG
forward primer with barcode 18	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCGATGTAAAACGACGGCCAG
forward primer with barcode 19	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACGATGTAAAACGACGGCCAG
forward primer with barcode 20	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCGATGTAAAACGACGGCCAG
forward primer with barcode 21	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACGATGTAAAACGACGGCCAG
forward primer with barcode 22	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCGATGTAAAACGACGGCCAG
forward primer with barcode 23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACGATGTAAAACGACGGCCAG
forward primer with barcode 24	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTATTTCGATGTAAAACGACGGCCAG
forward primer with barcode 25	CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACGATGTAAAACGACGGCCAG
forward primer with barcode 26	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCGATGTAAAACGACGGCCAG
forward primer with barcode 27	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCGATGTAAAACGACGGCCAG
forward primer with barcode 28	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCGATGTAAAACGACGGCCAG
forward primer with barcode 29	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCGATGTAAAACGACGGCCAG
forward primer with barcode 30	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTATCGATGTAAAACGACGGCCAG
forward primer with barcode 31	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCGATGTAAAACGACGGCCAG
forward primer with barcode 32	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACGATGTAAAACGACGGCCAG
forward primer with barcode 33	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTCATTGAACGATGTAAAACGACGGCCAG
forward primer with barcode 34	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCATCGTTTCGATGTAAAACGACGGCCAG
forward primer with barcode 35	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCCATTGTCGATGTAAAACGACGGCCAG
forward primer with barcode 36	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGAATCGTCGATGTAAAACGACGGCCAG
forward primer with barcode 37	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGAGAATGTCGATGTAAAACGACGGCCAG
forward primer with barcode 38	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGGACGGACGATGTAAAACGACGGCCAG
forward primer with barcode 39	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAATCGGCGATGTAAAACGACGGCCAG
forward primer with barcode 40	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACATAATCGATGTAAAACGACGGCCAG
forward primer with barcode 41	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCACTTCGCGATGTAAAACGACGGCCAG
forward primer with barcode 42	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACGAATCGATGTAAAACGACGGCCAG
forward primer with barcode 43	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGACACCGCGATGTAAAACGACGGCCAG
forward primer with barcode 44	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGGCCAGCGATGTAAAACGACGGCCAG
forward primer with barcode 45	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGCTTCTTCGATGTAAAACGACGGCCAG
forward primer with barcode 46	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGTCCGAACGATGTAAAACGACGGCCAG
forward primer with barcode 47	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGCAACCACGATGTAAAACGACGGCCAG
forward primer with barcode 48	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTAAGAGACGATGTAAAACGACGGCCAG
forward primer with barcode 49	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTAACATAACGATGTAAAACGACGGCCAG
forward primer with barcode 50	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGGACAATGGCGATGTAAAACGACGGCCAG

Table 2: Primers for 2nd PCR. Red: universal adaptor; black: index (barcode) sequence; blue: reverse sequencing adaptor; green: forward sequencing adaptor.

1 st PCR		
<i>Reaction conditions</i>		
Buffer: ABI PCR Buffer II 10x		
25 µl final volume		
500 ng DNA (for MRD detection and repertoire analysis) or 50ng DNA (for target screening)		
2,5 pmol of each primer		
dNTP: 5nmol		
MgCl ₂ : 37,5nmol		
Taq enzyme: 1U		
<i>Cycling conditions</i>		
Preactivation	94°C	10min
Denaturation	94°C	1min
Annealing	63°C	1min
Extension	72°C	30s
Final extension	72°C	30min
Number of cycles: 25		
Hold 4°C		

2 nd PCR		
<i>Reaction conditions</i>		
Buffer: FastStart High Fidelity Reaction Buffer, 10x concentrated, with 18mM MgCl ₂		
25 µl final volume		
1 µl 50x diluted 1st round product, purified with Agencourt AMPure Beads		
5 pmol of each primer		
dNTP: 5nmol		
FastStart High fidelity polymerase: 2,5U		
<i>Cycling conditions</i>		
Preactivation	95°C	2min
Denaturation	95°C	30s
Annealing	63°C	30s
Extension	72°C	30s
Final extension	72°C	5min
Number of cycles: 25		
Hold 4°C		

Table3: Reaction and cycling conditions for sequencing library preparation.

After the second round of PCR libraries were diluted 50 times and their quality and quantity was checked using Agilent High Sensitivity DNA kit. Libraries were then pooled equimolarly and the final pool was diluted to 20 pM (for sequencing using Ion Torrent PGM Sequencer) or to 11 pM (for sequencing using Ion Proton Sequencer) concentration prior the template preparation. The example of analysis of the final IgH and TRG libraries by Agilent 2100 Bioanalyzer is shown in Figure 3.

4.2.1 Primer sets for library preparation

Complete IgH gene rearrangements

Each VH segment contains three framework regions (FR1-FR3) which are similar among various VH segments and two complementarity-determining regions (CDR1

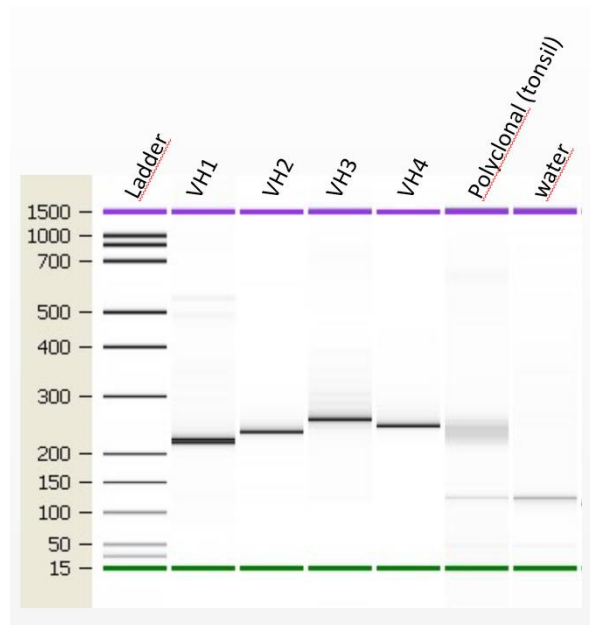


Figure 3: IgH libraries analysed by Agilent 2100 Bioanalyzer. Samples from patients with monoclonal rearrangements in respective V-segments were analysed.

and CDR2) which are highly variable (Figure 4). Framework regions are less frequently affected by somatic hypermutations and are thus an optimal primer targets.

The primers for IgH libraries preparation were derived from Biomed-2 primers that have been developed by the Euroclonality consortium for the detection of clonality in lymphoproliferative diseases⁵⁰. We used 7 VH-FR3 primers in conjunction with a single consensus JH primer which anneals to the homologous 3'-end regions of all JH segments. This set of primers should amplify virtually all possible V/J segments combinations. The JH primer can also be used for amplification of incomplete DH-JH rearrangements. Universal M13 adaptors which are complementary to the second round primers were added to the 5'-ends of the VH and JH primers.

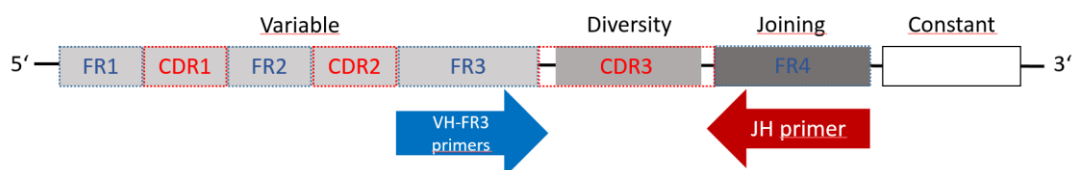


Figure 4: The scheme of IgH rearrangement with FR regions and CDRs. The localizations of primers are shown as arrows.

Incomplete IgH rearrangements

Rearrangements between DH and JH segments occur in early precursor B-cells and lead to the formation of incomplete DH-JH joints. These joints can be detected also in mature B-cell malignancies. Unlike VH-JH rearrangements, DH-JH rearrangements are usually not affected by somatic hypermutations⁶⁵ and this is very beneficial for the clone detection in B-cell proliferations with frequent somatic hypermutations.

The primers for incomplete IgH libraries were derived from primers presented by Szczepanski et al⁶⁶: seven family-specific DH primers were used together with the consensus JH primer which was already described in the section regarding complete IgH rearrangements. We modified primers for DH5 and DH7 families in order to obtain shorter PCR products which are easier to sequence. All DH primers were located upstream of the involved RSS, preventing the amplification of the D-J joint sequence in the cells with complete IgH rearrangements. 5'-ends of all primers were modified by adding universal M13 adaptors.

TRG rearrangements

The human TRG locus is localized on chromosome 7p14 and contains five J segments and 10 V segments which undergo the rearrangement process. The TRG locus does not contain D segments and the N-nucleotides additions are limited. Due to these reasons and limited number of V and J segments the junctional diversity in this locus is not very high. Rearranged TRG locus is present in both TR α β and TR γ δ lymphocytes, because unlike TRD, TRG is not deleted in TR α β cells.

For amplification of rearranged TRG genes we designed our own primer set consisting of four reverse J γ primers (the common primer for highly homologous J1 and J2 segments was used) and eight forward V γ primers, some of which were again common for sequentially homologous V segments. Again, the 5'-ends of the primers harboured universal M13 adaptors.

We used 22 diagnostic BM samples from patients whose malignant clones carried various combinations of all V γ and J γ segments in their TRG locus for testing

our primer set. We proved that all of the rearrangements can be amplified using our set of primers.

TRD rearrangements

The TRD locus is located between V α and J α gene segments and consists of 8 V δ , 4 J δ and 3 D δ segments. Rearrangements of TRD genes are one of the earliest events in the T-cell development. Five of the V δ segments can also rearrange to J α gene segments and V α segments can rarely form rearrangements with the segments from TRD locus. Despite the small number of V, D and J segments in this locus, the junctional diversity is high due to the addition of N-bases, random deletions and the fact that up to three D segments can form the junction (which can contain up to four N regions as a consequence).

For preparing TRD libraries we used modified classical Biomed-2 primer set⁵⁰ consisting of: six V δ primers, four J δ primers, D δ 2 primer (localized upstream of the D δ 2 gene segment) and D δ 3 primer (localized downstream of the D δ 3 gene

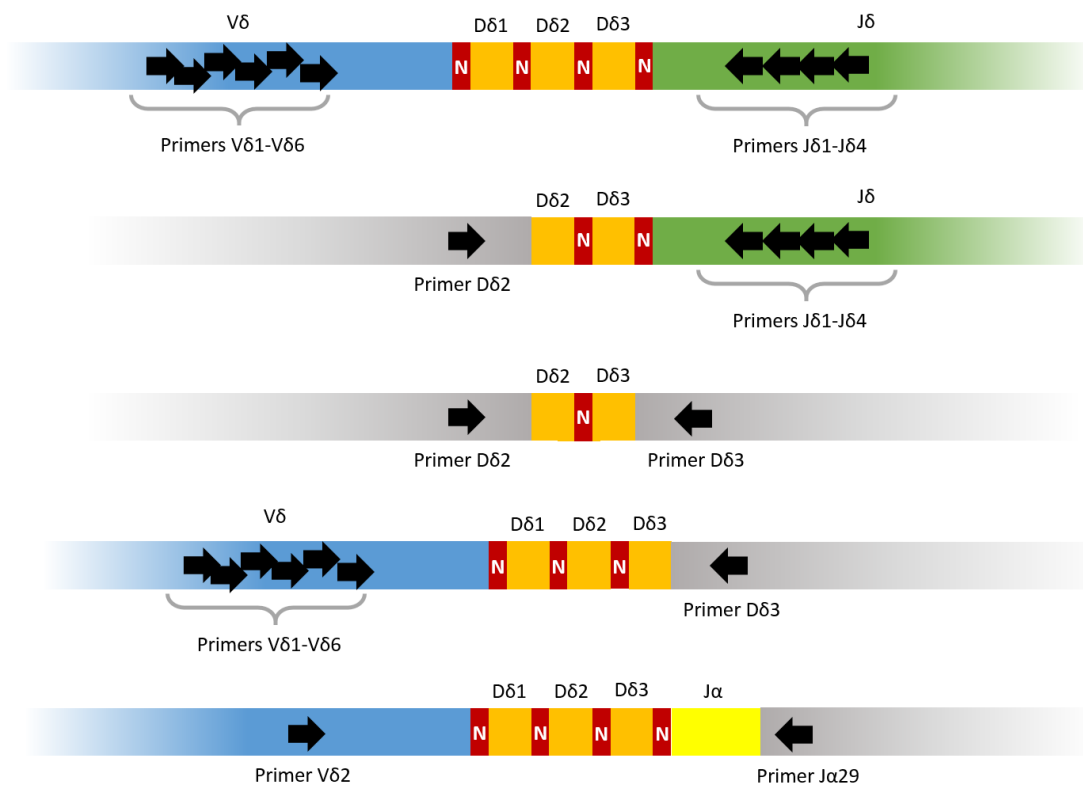


Figure 5: TRA/TRD locus PCR products.

segment). We added J α 29 primer presented by Szczepanski et al.⁴³, which should (in combination with V δ 2 Biomed-2 primer) amplify V δ 2-J α 29 joinings present in 42% of precursor B-ALL. The scheme of all potential PCR products resulting from PCR amplification using the above-mentioned primers is shown in Figure 5.

4.2.2 EuroClonality-NGS primer sets

EuroClonality-NGS (EC-NGS) Consortium on Next Generation Sequencing for Ig/TR immunogenetic analysis was established in 2012 with the aim to develop, standardize and validate Ig/TR NGS assays for the applications in hemato-oncology. The consortium is scientifically independent and works in a platform-independent way. Our laboratory centre is a member of the Consortium and participates in its activities.

The consortium is currently developing the assays for NGS of the rearrangements of IgH (both complete and incomplete), IgK, TR beta (TRB, both complete and incomplete), TRG, and TRD genes.

EC-NGS primers for the amplification of the IgH locus are localized in the FR1 and FR2 regions, unlike our in-house primers which are complementary to the sequences in FR3 region. The EC-NGS consortium decided to employ 1-step PCR

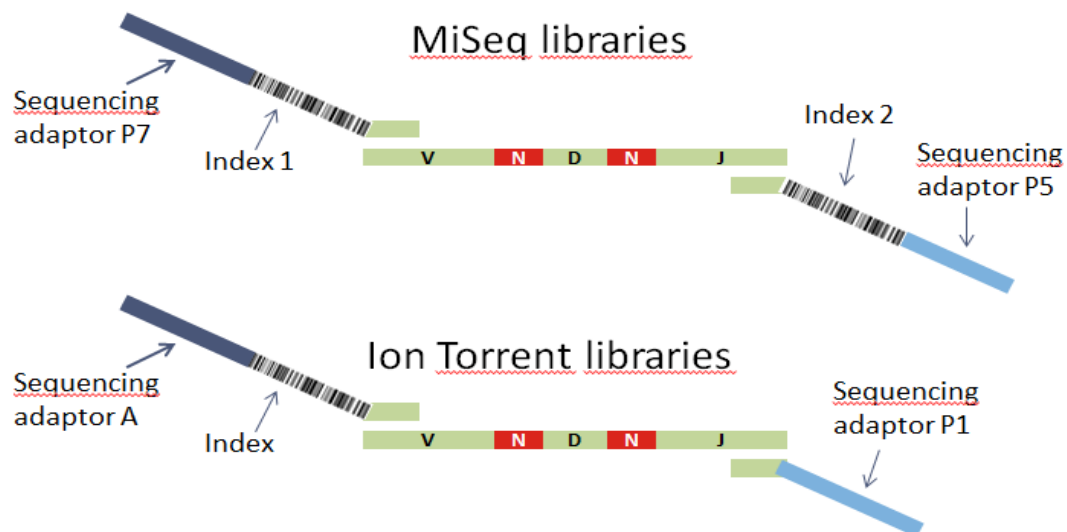


Figure 6: The EuroClonality-NGS library preparation strategy for Ion Torrent and Illumina.

approach for library preparation in order to prevent contaminations resulting from 2-round PCR approach.

The sequences of EC-NGS primers and the PCR conditions are confidential until published. The library preparation strategy for two leading sequencing platforms (Ion Torrent and Illumina sequencers) is shown in Figure 6.

4.3 Template preparation and Sequencing

The sequencing template was prepared via the emulsion PCR using the Ion OneTouch™ 2 Instrument and Ion PGM™ Template OT2 200 Kit or Ion PI™ Template OT2 200 Kit v2 according to the manufacturer's instructions.

After emulsion PCR the quality of the template was checked using the Ion Sphere Quality Control Kit and Qubit® 2.0 Fluorometer. If the percentage of Ion Sphere particles carrying the library molecules was between 10% and 30%, we proceeded to the template enrichment. Otherwise the template preparation was repeated with the modified library concentration.

It is very important to reach the correct percentage of templated Ion Sphere particles after emulsion PCR. Too high percentage means that too many Ion Sphere particles carry more than one library molecule before emulsion PCR and this results in polyclonal reads, which must be discarded. On the other hand too low percentage would result in a low chip loading and subsequently in a low sequencing output.

Enrichment of the template was performed using Ion OneTouch™ ES (enrichment system). During enrichment the empty Ion Sphere particles (carrying no library molecules) are removed from the template in order to reach high chip loading.

Sequencing was performed on Ion Torrent PGM or Ion Proton sequencers, using Ion PGM™ Sequencing 200 Kit v2 with Ion 318™ Chip Kit v2 or Ion PI™ Sequencing 200 Kit v2 with Ion PI™ Chip Kit v2 respectively. The sequencing outputs of such runs are ~5 million reads for Ion Torrent PGM and ~80 million reads for Ion Proton. Therefore we sequenced 5 samples within one PGM run and 50 samples within one Proton run.

DH-JH and TRD loci library fragments are too long for sequencing with 200bp kits. These libraries were processed using Ion PGM™ Template OT2 400 Kit and Ion PGM™ Sequencing 400 Kit (for Ion Proton 400bp kits are not available).

4.4 Data analysis

4.4.1 Primary Data Analysis Workflow

The raw sequencing data (in .dat format) were subjected to primary analysis workflow performed by the Torrent Suite™ Software (version 4.2), consisting of these steps:

1. signal processing – generating raw incorporation signal (pH change) measure for each single well on the chip (resulting in the .wells files)
2. base calling – generating the nucleotide sequence for each well (in .sff format)
3. reads filtering – filtering of polyclonal (mixed-template) reads, low quality reads and reads resulting from primer-dimers
4. reads trimming – adapters trimming and 3' quality trimming
5. demultiplexing – assignment of reads to samples based on their barcodes

Fastq⁶⁷ files for all separate barcodes were created using the Fastq Creator plugin (version 3.4.56313) for Torrent Server. Fastq file is text-based and stores base calls (nucleotide sequences) together with their associated quality scores (in Phred scale), both encoded by a single ASCII character.

4.4.2 Identification of CDR3 regions in diagnostic samples

The rearranged Ig/TR sequences of the diagnostic clones were obtained by PCR amplification of the diagnostic bone marrow DNA with Biomed-1⁶⁸ primers and subsequent Sanger sequencing of resulting PCR products.

All the sequences were then analysed with the online IMGT/V-QUEST tool⁶⁹ (<http://www.imgt.org>), which identifies V, D and J segments of each rearrangement by alignment to the germline Ig and TR sequences.

4.4.3 MRD detection in fastq files

Reads shorter than 100bp were filtered out of the fastq files using the Usearch⁷⁰ sequence analysis tool. The length of such reads is not sufficient as they do not reach the junction, which is crucial for the correct identification of the diagnostic sequence.

Because Ion Torrent data contain a significant number of errors ($\sim 1.7\%^{16}$) which could hamper the detection of the sequence from diagnosis, we decided to reduce this risk by searching for short (15bp) markers spanning the V-D/D-J/V-J junctions.

For each rearrangement we defined more markers (their number depends on the number and length of N-segments), which were shifted against each other by two nucleotides. Taken together these markers covered the entire junction region. The strategy for markers selection is shown in Figure 7.

The fastq files from each patient were then searched for markers covering his rearrangement(s) using the fqgrep tool (<https://github.com/indraniel/fqgrep>), which is a sequence pattern matcher optimized for fastq/fasta files. For each rearrangement the numbers of matches for all markers were checked for large discrepancies and such discrepant markers were excluded. MRD was then expressed as the average number of matches per marker per total number of reads. In cases where polyclonal DNA was added before library preparation the total number of reads was reduced by 10% before the calculation of MRD.

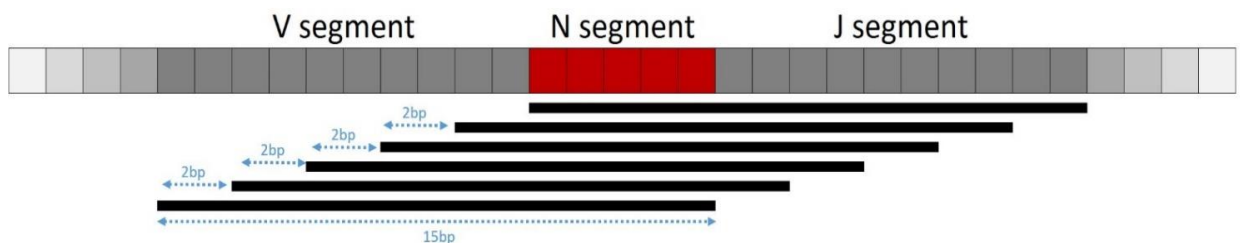


Figure 7: The strategy for markers selection. Markers are represented by black lines.

4.4.4 The Assessment of repertoire diversity and detection of diagnostic leukaemia-associated clones

The fastq files were uploaded to the online open-source program called Vidjil⁷¹ (version 2015.04, <http://www.vidjil.org>). Its algorithm is designed for NGS data and it quantifies the clonotypes abundances. Because it only focuses on the sequences of the recombinant junctions and not on the entire reads, the algorithm is fast and can process large datasets in a few minutes. Vidjil employs the IMGT/GENE-DB database⁷² of germline genes.

In the first stage Vidjil extracts V(D)J junctions from sequences in the fastq files and defines short zones called w-windows, which are overlapping the third complementarity determining region (CRD3). The positions of w-windows are determined by the germline V and J segments which are closest to the junction. This step is followed by identification and clusterization of the clones. Reads are clustered into a clone only in case the sequence of their w-window covering the CRD3 region is identical (no mismatches are allowed). The representative sequence for each clone is then computed and it is further processed to obtain its full V(D)J segmentation and other useful information.

The data generated by all NGS platforms contain a substantial number of errors, which might lead to the false detection of extra clones. Because our data were all produced by the same sequencer, the error rate should be the same for all the data and the number of clones generated by sequencing errors should be therefore related to the total number of reads. In Figure 8 you can see the high correlation ($R^2=0.94$) of the number of reads and the number of identified clonotypes in 149 samples from patients with ALL (74 samples from day 33 and 75 samples from day 78) which is consistent with the previous assumption. We therefore decided to express the repertoire diversity as the clones/total reads ratio.

We used Vidjil also for the detection of leukaemic clones in diagnostic samples, which were further used for patient-specific qPCR primers design. The detection of these clones is not very challenging because they are represented by a high

proportion of reads. In case of polyclonality or biallelic rearrangements the Vidjil platform enables the detection of all present clonotypes.

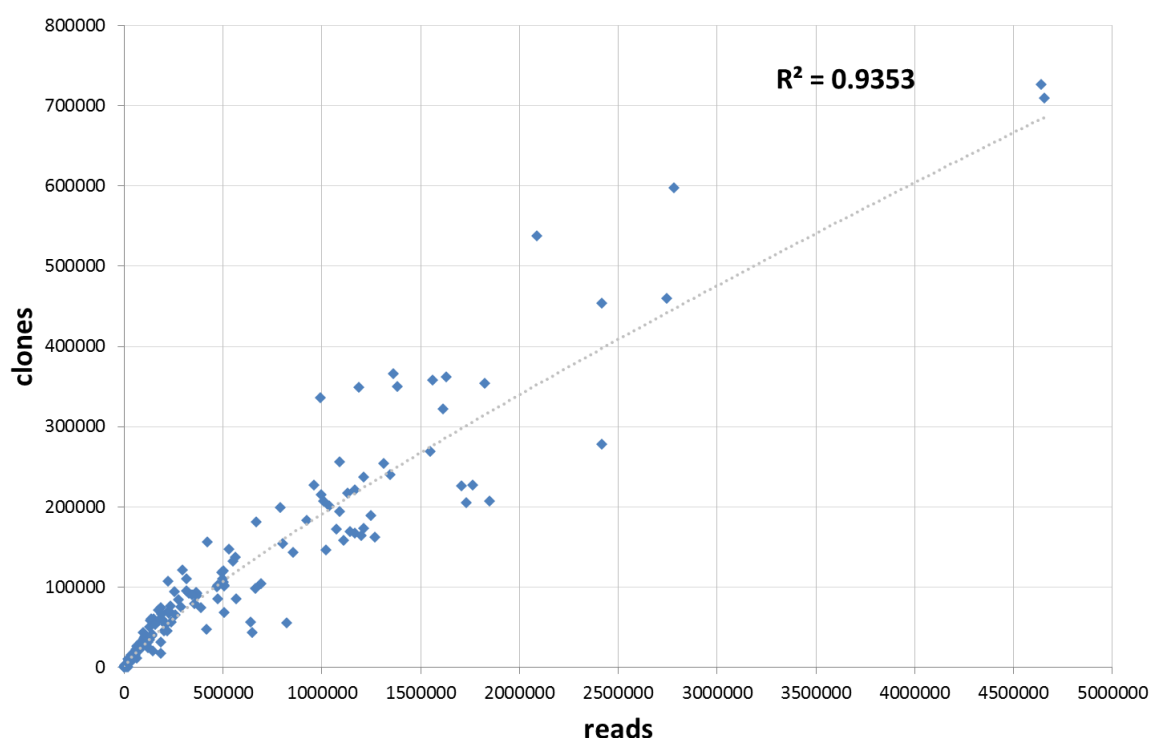


Figure 8: The correlation of number of reads per sample with the number of clones in 149 samples.

4.4.5 Validation of the assay for MRD detection

To test the sensitivity and quantitative range of our NGS assay for MRD detection we sequenced several multiplicates of six different dilutions of two diagnostic ALL samples with two different IgH rearrangements (VH2-DH6-JH4 and VH1-DH3-JH4).

The 10^{-1} to 10^{-5} dilutions were prepared by diluting the diagnostic sample into the DNA from mononuclear cells of healthy donors (buffy coat, BC) logarithmically. 10^{-1} , 10^{-2} and 10^{-3} dilutions were sequenced in duplicates and 5×10^{-4} , 10^{-4} and 10^{-5} dilutions in quadruplicates with the median coverage of 401,802 reads per library. Buffy coat in monoplicate was added as a negative control.

The results of the dilution experiment are shown in Figure 9. They are reproducible up to 5×10^{-4} dilution and reach the sensitivity of 10^{-5} in 50% of replicates in both patients.

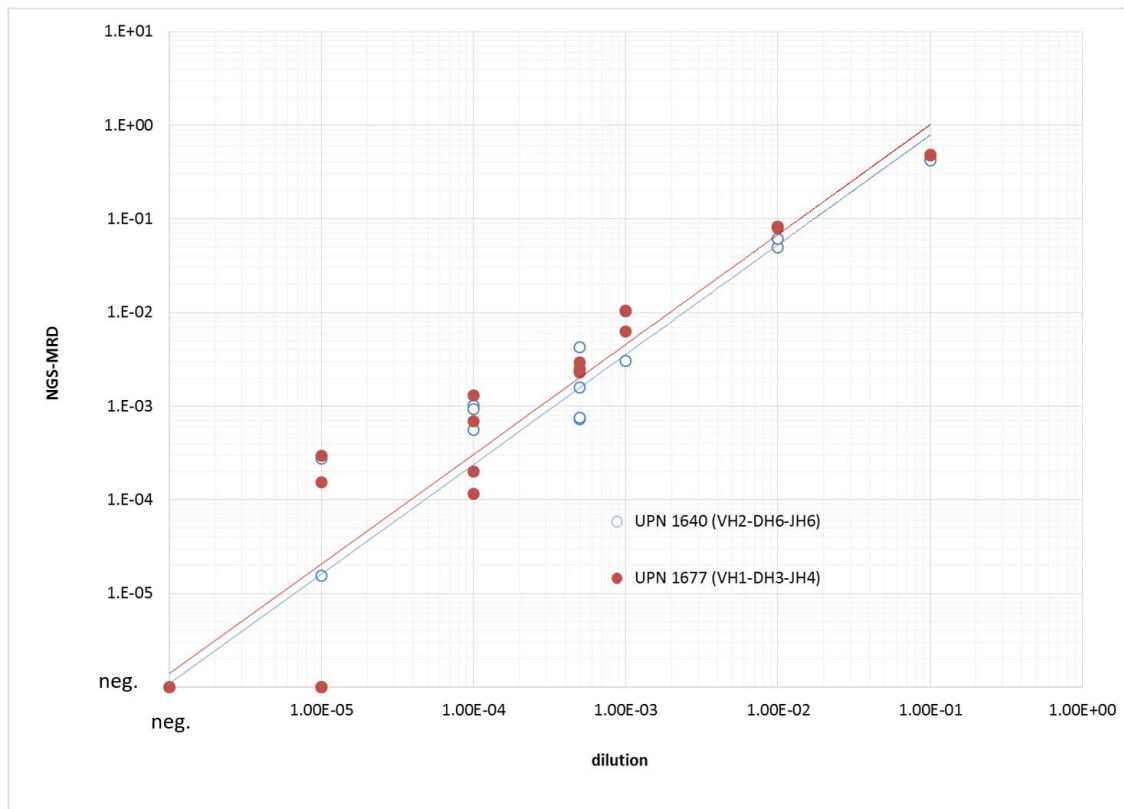


Figure 9: Results of the dilution experiment. The proportion of reads harbouring the diagnostic clonal IgH rearrangement is shown on the Y-axis, the dilution is shown on the X-axis. Figure from Kotrova et al., *Blood*, 2015.

5. Results

5.1 Target identification

It was already mentioned that identification of clonal rearrangement in the diagnostic sample is necessary for further qPCR-based tracking of the leukemic clone in the follow-up samples. Unfortunately in some patients target identification by classical multiplex PCR followed by Sanger sequencing of PCR products fails and thus precludes the MRD detection.

5.1.2 Target identification in mantle cell lymphoma

We performed NGS-based target screening in six patients with mantle cell lymphoma in whom the classical method failed due to the low infiltration of bone marrow at diagnosis.

Sequencing libraries (Figure 10) were prepared using the primer mix for complete IgH rearrangements, as described in the Methods section. All the six

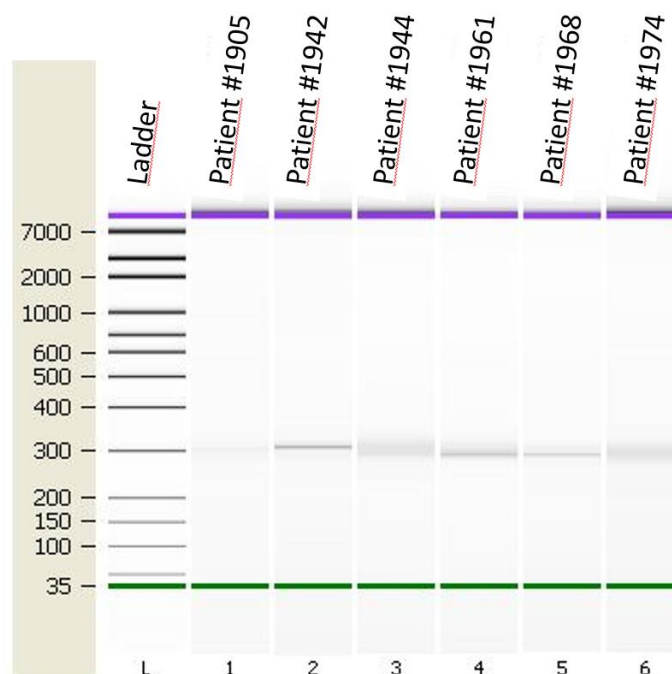


Figure 10: Sequencing libraries created from diagnostic DNA of six MCL patients analysed by Agilent 2100 Bioanalyzer.

libraries were sequenced with the median coverage of 32,096 reads per sample (range 25,871 – 45,498), the prevailing rearrangement was identified for all six patients and successfully used for subsequent qPCR MRD detection in samples from later time-points. We compared the proportion of reads representing the prevailing clone with the infiltration of bone marrow at diagnosis detected by flow cytometry: the strong correlation between these two values ($R^2=0.77$) has been proven (Figure 11).

5.1.3 Target identification in ALL

We utilized NGS-based IgH target screening also in two paediatric ALL patients in whom the classical approach was unsuccessful. In patient #2473 a clear band (Figure 12) was obtained via electrophoresis with the Agilent 2100 Bioanalyzer following the PCR reaction with IgH primer set. However, Sanger sequencing provided only a mixed sequence which was not usable for the design of patient-specific primers.

Using NGS we identified two prevailing rearrangements (Figure 13):

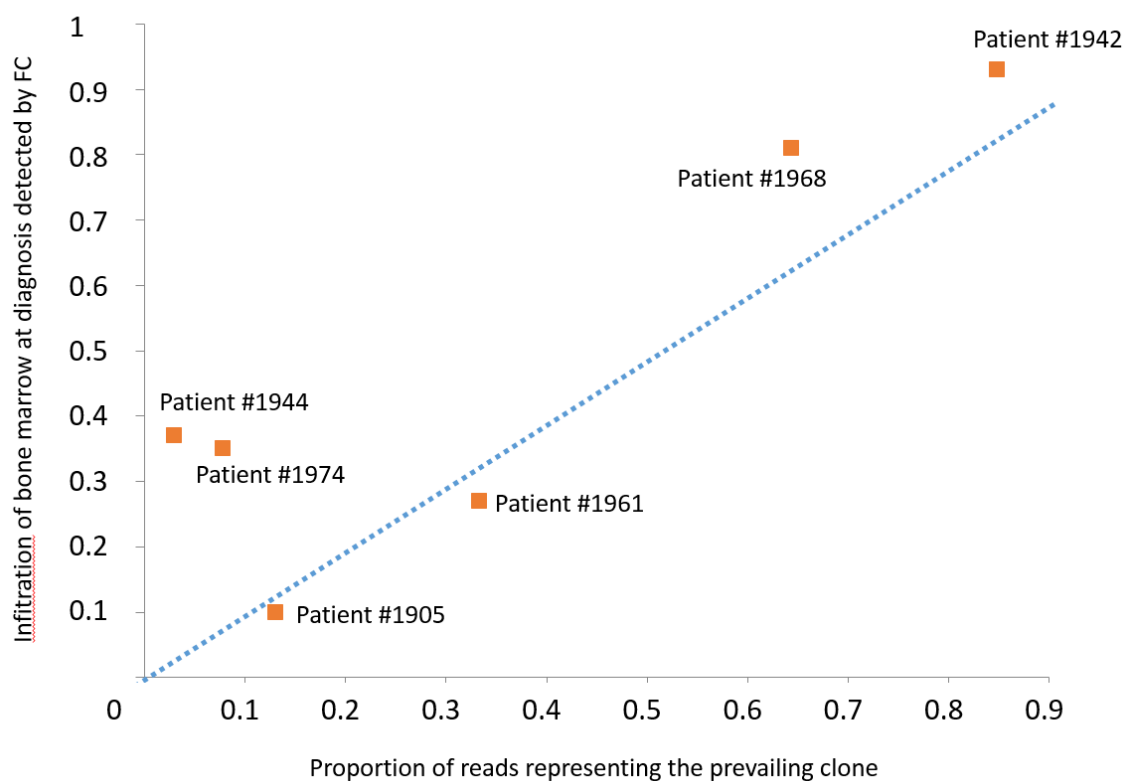


Figure 11: The correlation of the bone marrow infiltration at diagnosis and the proportion of reads representing the prevailing clone.

IGHV3.11-D5.24-J4 (represented by 51% of reads) and IGHV3.13-D4.11-J6 (represented by 21% of reads). The fact that both prevailing rearrangements contain a V segment from the V3 family (and are thus both amplified by the same forward primer) explains the presence of mixed sequence obtained by Sanger sequencing of PCR product generated through the reaction utilizing VH3 primer.

In the patient #2092 one rearrangement (IGHV4.39-D3.16-J6) represented by 80% of reads was detected by NGS, as well as by a classical approach (Figure 13). NGS detected also one additional rearrangement (IGHV1.NL1-D3.22-J3) which was represented only by a very weak band on Agilent 2100 Bioanalyzer Electrophoresis (Figure 12).

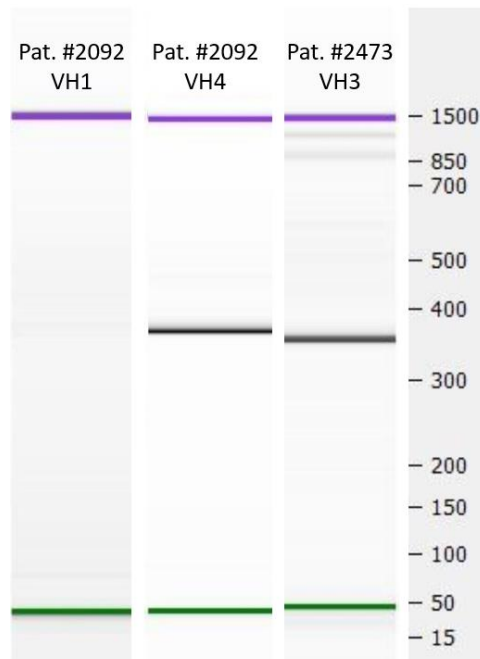


Figure 12: Agilent 2100 Bioanalyzer electropherogram of IgH PCR products from patients #2092 and #2473.

5.2 MRD detection at days 15, 33, and 78

We detected MRD by NGS in 210 samples from 76 children with B-precursor ALL (62 × day 15, 73 × day 33, 75 × day 78). Patients were selected based on the availability of at least one IgH (VH-JH) qPCR target fulfilling the EuroMRD criteria³⁷ for a sensitivity of at least 10^{-4} . Sixty-two patients were consecutive cases treated on AIEOP BFM-ALL 2000 protocol^{30,45}. In order to better describe the prognostic relevance of NGS-MRD we added fourteen consecutive patients

from previous (ALLIC-BFM 2002⁷³) and subsequent (AIEOP-BFM-ALL 2009) treatment protocols. The median coverage was 729,842 reads per sample.

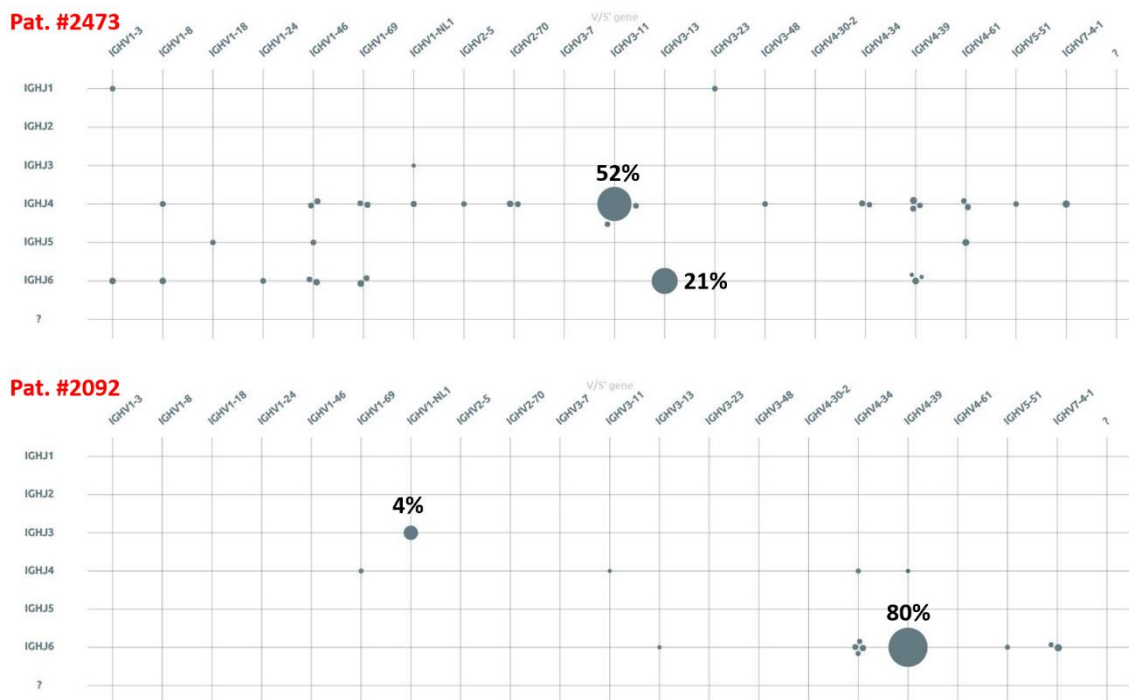


Figure 13: Representation of clones displayed by the Vidjil tool. Each dot represents a clone and the size of each dot reflects the size of the clone.

5.2.1 Correlation of MRD detected by qPCR and NGS

The comparison of MRD levels detected by NGS and by qPCR is shown in Figure 14: The overall correlation of both methods was high ($R^2=0.72$) with only 28 (13%) discrepant samples (15 and 13 samples were NGS positive, qPCR negative and NGS negative, qPCR positive respectively). Eighty-two samples (39%) were concordantly identified as negative by both methods.

The majority of the discrepant samples have MRD levels (detected either by qPCR or by NGS) below the sensitivity of both methods and therefore the discrepancies are not so surprising. The sensitivity of both NGS and qPCR cannot be higher than the number of analysed cells. We analysed approximately 70,000 cells by both methods and therefore we assume that their sensitivity cannot exceed 1 cell in 10^5 cells.

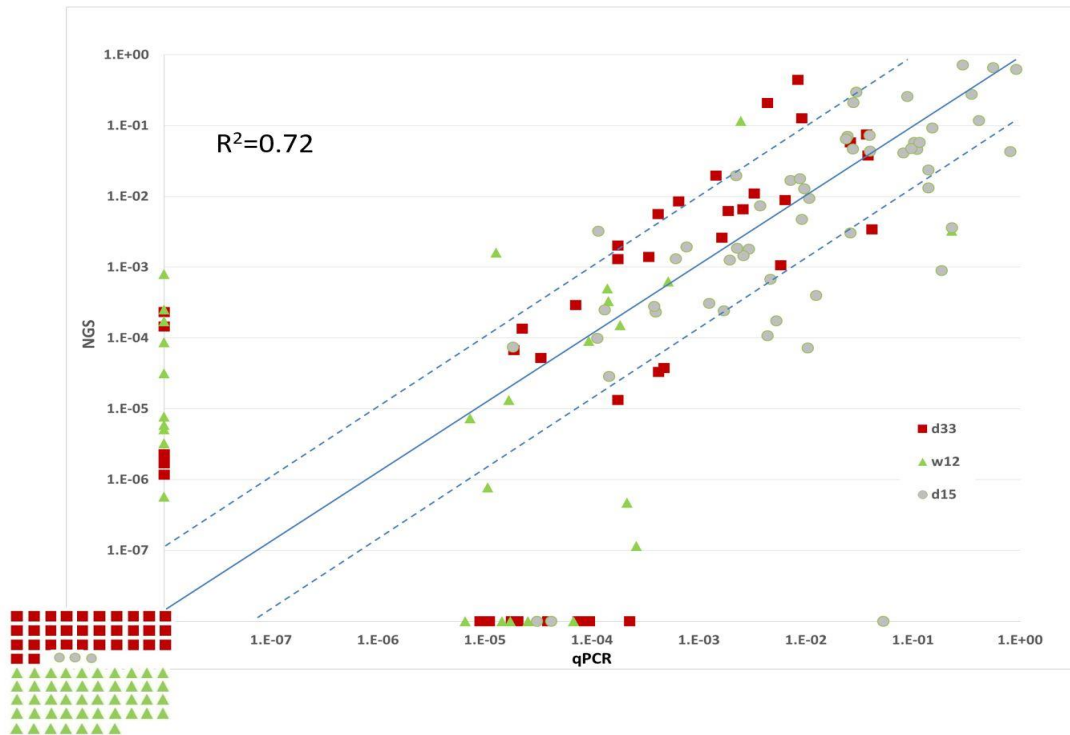


Figure 14: The comparison of MRD detected by NGS (Y-axis) and qPCR (X-axis) at days 15, 33 and 78. Figure from Kotrova et al., *Blood*, 2015.

5.2.2 Changes in the risk group stratification

We checked how the differences in MRD levels detected by NGS and by qPCR would affect the final BFM risk stratification described in the Introduction section (Figure 15). If NGS results were used for the stratification instead of the qPCR results, seven (9%) patients would be reassigned from SR to IR group, ten (13%) patients from IR to SR group, five (7%) patients would be shifted from IR to SER, two (3%) patients from SER to IR and one patient from SER to HR group.

Six out of 13 patients who would be reassigned into prognostically worse risk group by NGS relapsed, whereas there were only 2 relapses in 12 patients who would be reassigned into prognostically better risk group by NGS. This may be a hint that stratification based on NGS results is more reliable for the patients' stratification. Nearly one third of patients from SR and IR groups were not assigned consistently by NGS and qPCR. This is mainly caused by the non-consistent MRD results below the sensitivity of both assays, as discussed in the previous section.

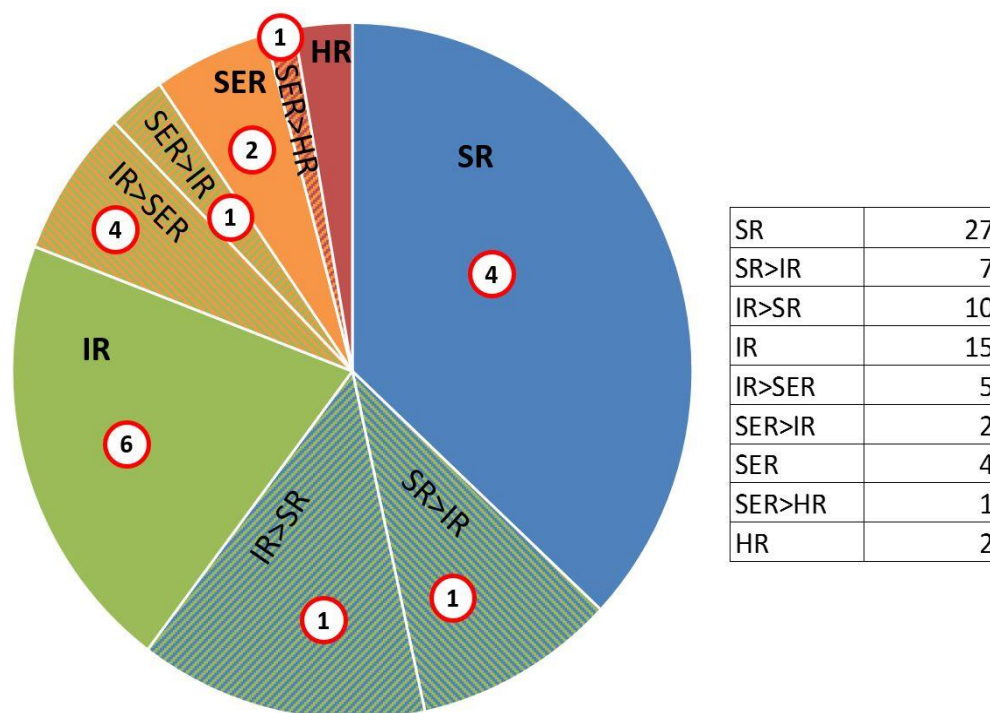


Figure 15: Differences in the risk group stratification if NGS results were used instead of qPCR results. Numbers in the red circles represent the number of relapses in each subgroup. The table on the left shows the number of patients in each subgroup.

5.2.3 NGS-MRD and the relapse prediction

We showed that the significant proportion of patients (13 out of 15; 87%) who subsequently relapsed had day 33 MRD levels detected by NGS higher than qPCR-MRD levels. Conversely, only 13 out of 25 (52%) patients without relapse had NGS-MRD higher than qPCR-MRD. Nine patients without relapse were NGS-negative, qPCR positive at day 33, with the qPCR values ranging between $8.98E-06$ and $2.26E-04$ (median $3.84E-05$). This suggests that these marginal qPCR-positive results might be false positivities and that NGS is more specific for relapse prediction than qPCR at day 33.

We did not observe the same trend within the MRD results from day 78. This is probably caused by the fact that a very large proportion of samples from this time-point is MRD-negative (75% and 69% of samples are negative by qPCR and NGS respectively).

The survival curves (Figure 16 A) for patients separated based on the MRD-negativity/positivity at day 33 NGS show that NGS defines a larger group of MRD-negative patients (41 patients negative by NGS vs. 37 by qPCR) which have a slightly better prognosis (5-year relapse-free survival, RFS =90±5%, vs. 53±9% in MRD-positive group, p=0.0003) than the group of qPCR negative patients (5-year RFS =84±6%, vs. 63±8% in MRD-positive subgroup, p=0.04). The prediction of RFS based on MRD results from day 78 is identical for both qPCR and NGS (5-year RFS for NGS negative subgroup is 82±5% vs. 57±10% for NGS positive subgroup, p=0.01; for qPCR negative subgroup RFS is 80±5% vs. 56±12% for qPCR positive subgroup, p=0.02, Figure 16 B). Using the combined day 33 and day 78 MRD levels for stratification of patients into the three risk groups based on BFM risk stratification criteria gives slightly better results for NGS, which are probably driven by days 33 results (RFS for NGS: SR 89±5%, IR 67±10%, HR 42±14%, p=0.002, RFS for qPCR: SR 85±6%, IR 66±9%, HR 56±17%, p=0.07, Figure 16 C). For the purposes of this analysis SER subgroup is included in the HR group.

Using NGS-MRD results from days 15 we were able to define a subgroup of patients with MRD $< 5 \times 10^{-4}$ and excellent prognosis (5-year RFS=89±7% vs. 64±7% in a subgroup with MRD $> 5 \times 10^{-4}$, p=0.05). The subgroups of similar sizes and prognosis were defined also by FC and qPCR (FC $< 10^{-3}$ vs. FC $\geq 10^{-3}$: 5-year RFS=100% vs. 63±7%, p=0.01, qPCR $< 5 \times 10^{-4}$ vs. qPCR $\geq 5 \times 10^{-4}$: 5-year RFS=92±8%, vs. 66±7%, p=0.09). This is consistent with previously published data about the excellent outcome of patients with very low MRD at day 15⁷⁴⁻⁷⁶.

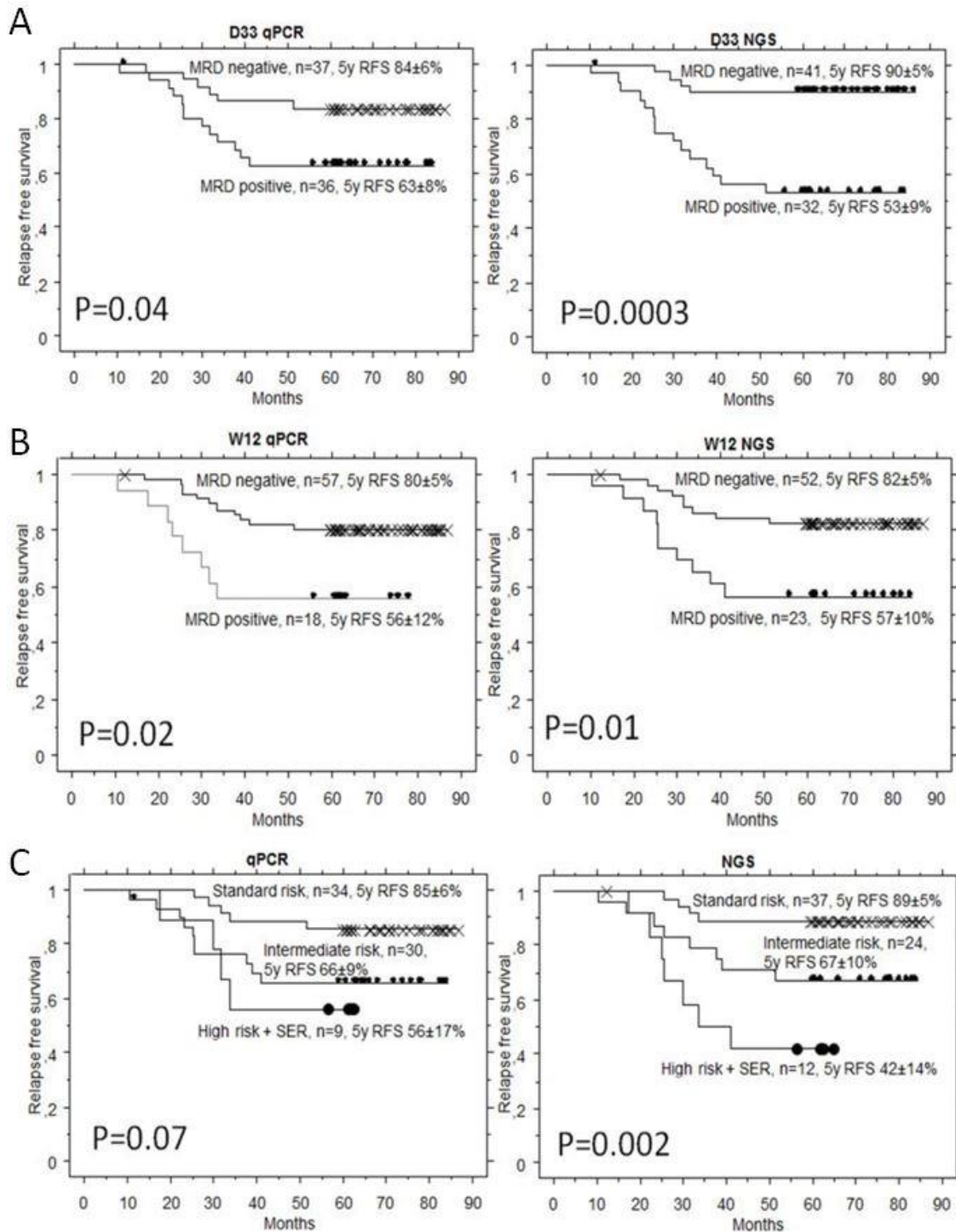


Figure 16: Kaplan-Meier survival curves showing relapse-free survival (RFS) of ALL patients stratified into the risk groups based on qPCR/NGS-MRD results. A) based on MRD negativity/positivity at day 33; B) based on MRD negativity/positivity at day 78 (week 12); C) based on BFM risk group stratification. Figure from Kotrova et al., Blood, 2015.

5.2.4 The correlation of MRD levels based on different targets

Twelve of the patients in our cohort had two different complete IgH (VH-JH) MRD targets. In these patients we compared MRD detected by NGS as well as by qPCR based on each of the targets (Figure 17).

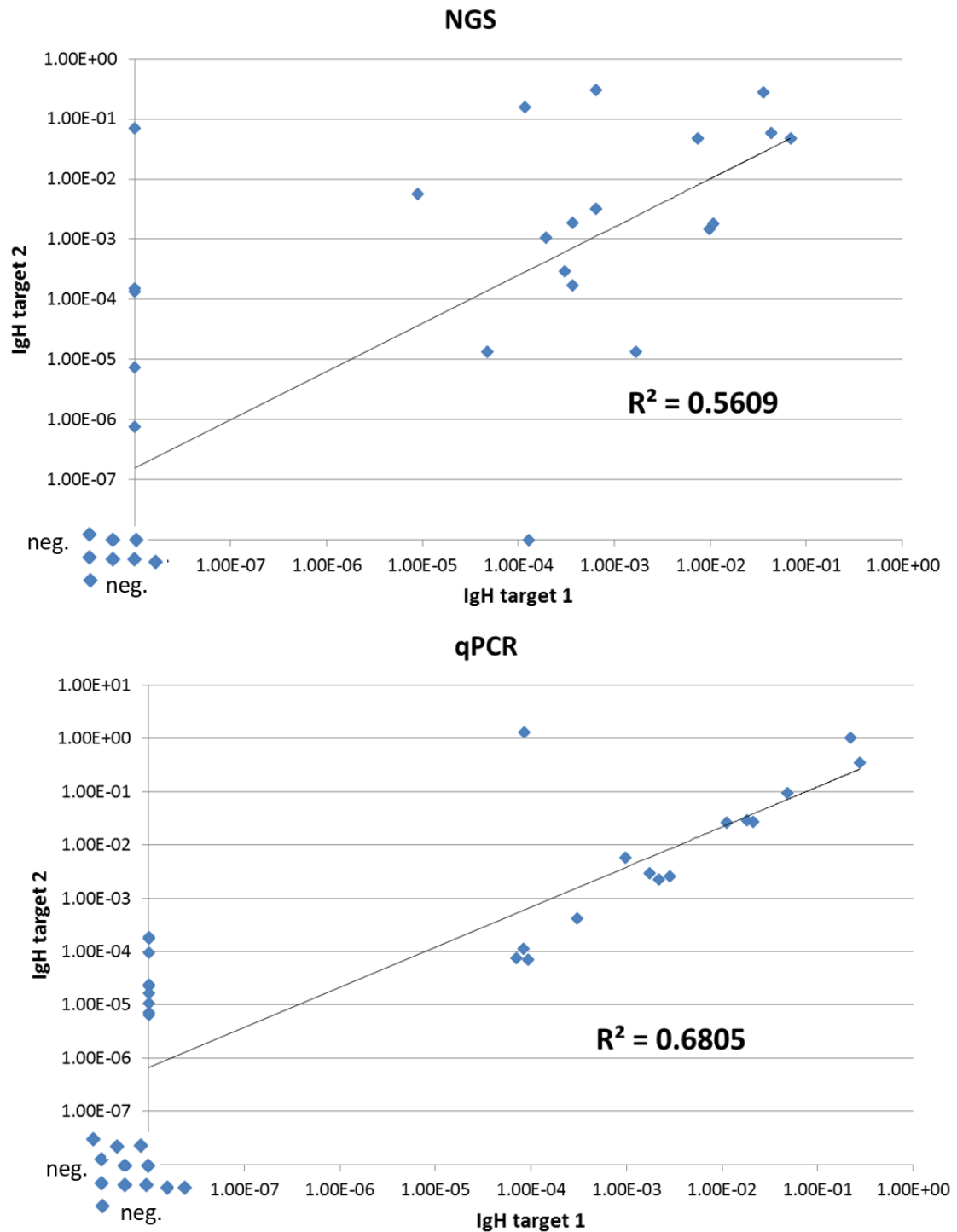


Figure 17: MRD levels based on two different targets as detected by NGS (upper graph) and qPCR (lower graph).

The correlation was better for qPCR, but it is important to note that MRD in the diagnostic samples is normalised to 100% for both targets in assays based on qPCR and that the follow-up samples are quantified against the dilution curves created from the diagnostic DNA. This artificial normalization may lead to the tighter correlation of qPCR results. On the other hand NGS shows the real representation of the target compared to the background.

Some of the non-correlating targets may be present in different leukemic subclones and may not be supposed to correlate in fact. Unfortunately all non-correlating rearrangements are unproductive and therefore it is unclear whether they can be present in a single cell or not.

5.3 Repertoire diversity

We performed the assessment of repertoire diversity in samples from days 33 and 78 (the detailed explanation of the methodology is provided in the Methods section) in our cohort of B-precursor ALL patients. We excluded samples with less than 10,000 reads successfully assessed by Vidjil, because our data show that the clones/reads ratio in the samples with low coverage is skewed (Figure 18). This is

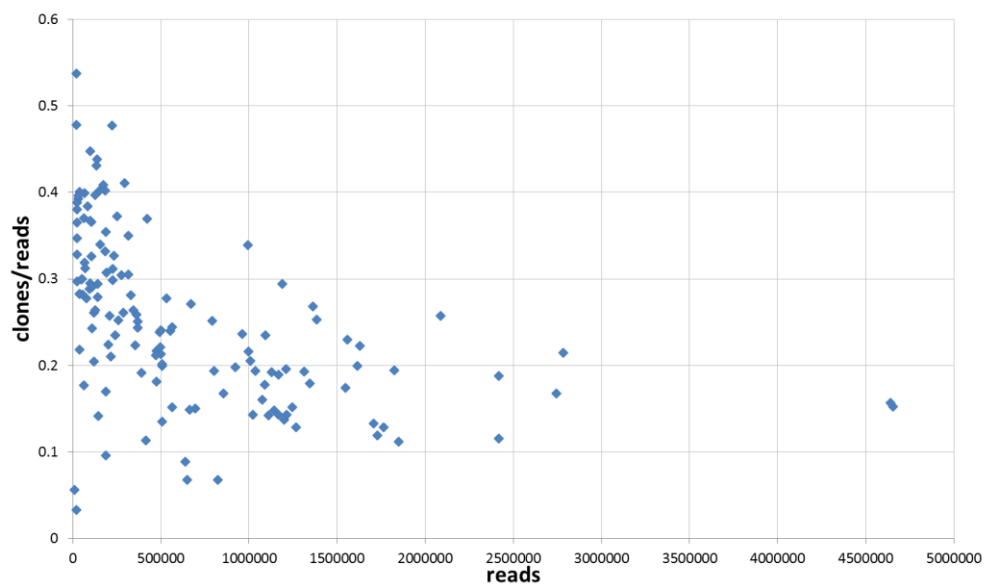


Figure 18: The dependence of clones/reads ratio (Y-axis) on the total number of reads per sample (X-axis). In samples with low coverage the ratio is skewed.

probably caused by the fact that the number of clones in these samples highly exceeds the number of reads and therefore almost every read represents a different clone.

Altogether we analysed 69 day 33 samples and 71 day 78 samples with more than 10,000 reads assessed by Vidjil. Although it was clear that clones/reads ratio is independent of MRD (Figure 19) and thereby that the presence of leukemic clone does not affect the repertoire diversity, we decided to exclude samples with the MRD above 10^{-4} from further analyses.

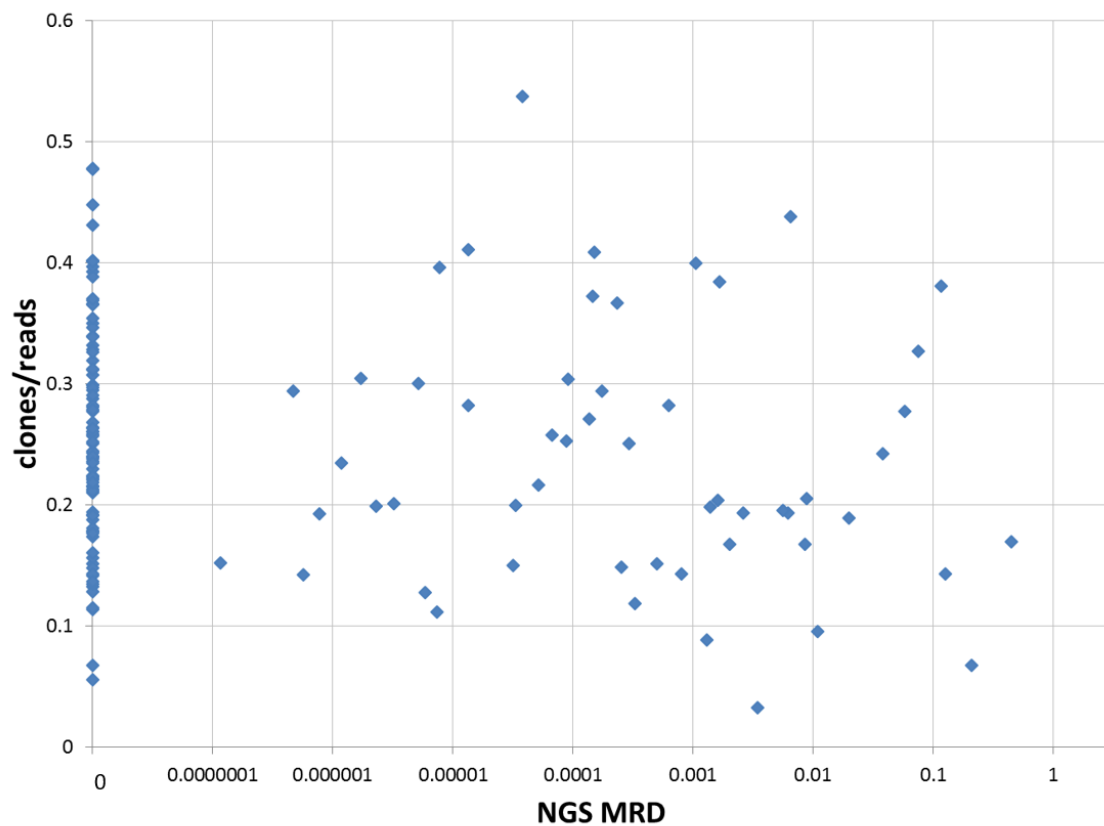


Figure 19: Clones/reads ratio (Y-axis) is independent of MRD (X-axis).

We compared the repertoire diversity (clones/reads ratio) between patients with and without subsequent relapse at day 78 (Figure 20). The repertoire diversity in samples from patients without subsequent relapse was significantly higher ($p=0.005$). At day 33 the difference between patients with and without relapse was not significant, probably due to the small number of relapsed patients with MRD level below 10^{-4} at this time-point.

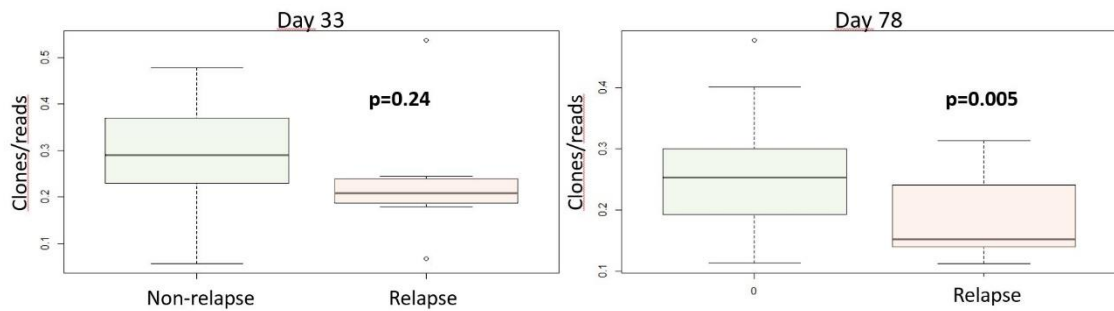


Figure 20: Repertoire diversity (represented by clones/reads ratio) in patients with and without relapse at days 33 and 78.

We also showed that clones/reads ratio at day 78 has a prognostic significance. Patients with higher clones/reads ratio (> 0.18) have a 5-year RFS $88 \pm 5\%$, whereas patients with clones/reads ratio < 0.18 have 5-year RFS only $50 \pm 12\%$ ($p=0.0005$, Figure 21).

Nineteen out of 20 patients with relapse in this study had the material from day 78 available. Thirteen out of this 19 patients (68%) had the clones/reads ratio at day 78 below the threshold of 0.18, four of whom were MRD-negative (by NGS as well as qPCR) at both days 33 and 78 and thus were assigned to standard risk group.

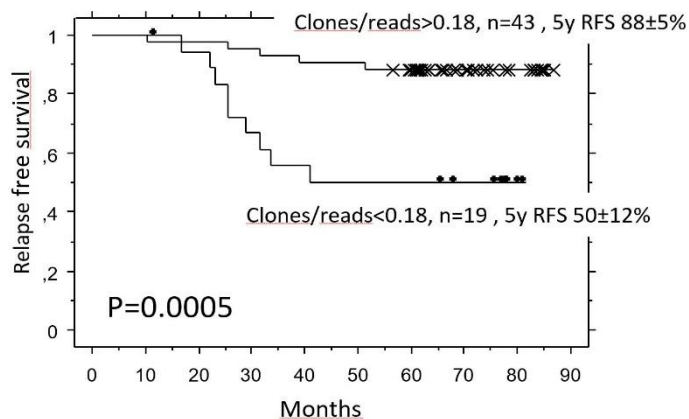


Figure 21: Relapse—free survival (RFS) in patients with high (>0.18) and low (<0.18) clones/reads ratio at day 78. Figure from Kotrova et al., Blood, 2015.

5.4 False positivities in post-transplant samples

We have previously shown that MRD detection based on qPCR for Ig/TR rearrangements can yield false positive results in patients after hematopoietic stem cell transplantation. We supposed that these results were caused by unspecific binding of clone-specific qPCR primers due to massive regeneration of B-cells⁴⁷. To prove that

the qPCR-positive results in these patients are really false-positive and to find the source of the unspecific amplification, we selected 34 samples from 21 paediatric B-cell precursor ALL patients after HSCT, which were low-positive (not quantifiable) by qPCR. All patients remained in complete remission after HSCT, despite the fact that no therapy intensification was implemented, except for one of them, who received three donor lymphocyte infusions (DLI) based on qPCR positivity. The median follow-up in the cohort was 10.4 years (range 2-15.5). Six low qPCR positive post-transplant samples from the patients who later developed relapses were added as the positive controls.

The library preparation and sequencing approach are described in the Methods section. Twenty-three patients carried the complete IgH (VH-JH) target in their leukemic clone, 3 patients the TRG target and 2 patients incomplete IgH (DH-JH) target. The median coverage was over 1 million reads per sample.

5.4.1 MRD detection

Only 2 of 34 (6%) follow-up samples from patients without the subsequent relapse were found to be NGS-MRD-positive, defined as the presence of leukaemia-specific sequence(s) as identified at diagnosis in the follow-up samples. One of the positive samples belonged to the patient with the shortest follow-up (2 years) and unclear outcome so far.

Both samples which were positive by NGS harboured the complete IgH (VH-JH) target. One of these samples was surprisingly highly positive by NGS (MRD level was 10^{-3}) despite the fact, that the MRD level detected by qPCR was below the quantitative range (which was 10^{-4}). This quantitative discrepancy can be caused by the overamplification of leukaemia-specific sequence on the background of the B-cell aplasia (we did not add 10% of BC to normalize the quantitative levels, because our initial goal was only to distinguish between positive and negative samples). The second sample was MRD-positive on the level 5×10^{-5} which is in accordance with the non-quantifiable qPCR result (quantitative range was 5×10^{-4}). Surprisingly, this was a sample from peripheral blood and the bone marrow sample from the same

time-point was negative by NGS (although not quantifiably positive by qPCR). All the remaining samples from patients without subsequent relapse were negative by NGS⁷⁷.

We added six samples with low-level qPCR positivities from patients who subsequently relapsed as controls. Four (66%) of these samples were positive also by NGS. All the NGS-positive results were at least 1 log higher than qPCR positivities which was probably caused by the same mechanism as in one of the positive patients without relapse.

5.4.2 Identification of the source of unspecific amplification

To find the source of the unspecific amplification leading to supposedly false positive qPCR results we searched (using the `fqgrep` tool) for matches of the clone-specific qPCR primers sequences in all fastq files resulting from the sequencing of the libraries which were created using the same set of primers (IgH VH-JH/IgH DH-JH/TRG).

For all primers, similar sequences (with maximally 2 mismatched nucleotide bases) were found in fastq files from different patients, thus likely explaining false positivities by qPCR analysis.

5.5 Clonal evolution during the course of the disease

In the B-precursor ALL patient #1256 we identified the VH3-JH rearrangement at diagnosis by the classical screening consisting of the multiplex PCR using consensus primers followed by Sanger sequencing of the resulting PCR product.

We sequenced IgH libraries created from this patients' samples taken at days 15, 33, and 78 and analysed the results using the `Vidjil` tool. At day 15 we identified two prevailing IgH clones: the VH3.11-DH3.22-JH4 clone which was already identified at diagnosis and a new clone VH4.34-DH3.22-JH4. At day 33 (Figure 22) the new clone VH7.40-DH3.22-JH4 appeared.

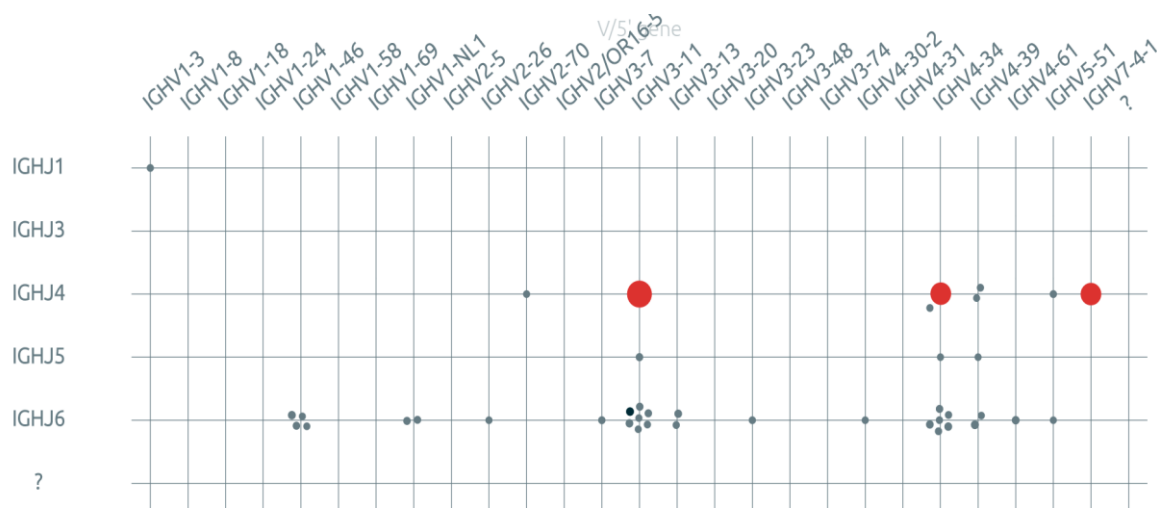


Figure 22: Leukaemia clones evolved via clonal evolution in patient #1256 at day 33. Visualization was done by the Vidjil tool.

These three clones harbour the same D-J stem and different V segments (Figure 23), thus apparently result from the clonal evolution process which was described in the Introduction section. Classical qPCR method would not detect the newly evolved clones in case that the clone-specific primers were located in V-D region.

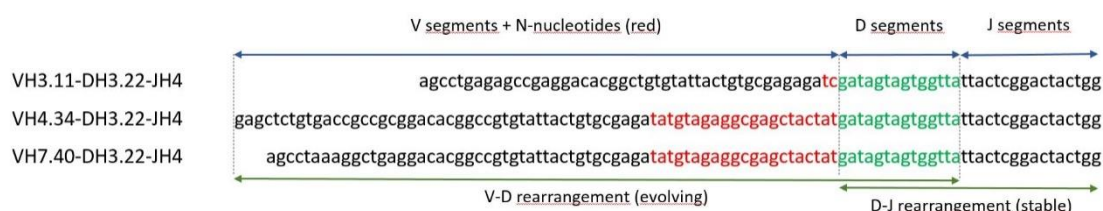


Figure 23: Sequences of the IgH locus of the three related clones in patient 1256.

5.6 The comparison of two sequencing platforms

In order to compare the performance of the EC-NGS one-step IGH PCR assay on two different sequencing platforms we sequenced serial dilutions (10^{-1} , 10^{-3} , 10^{-4} , and 10^{-5}) of 5 diagnostic ALL samples (one of the samples harboured two IgH targets) together with polyclonal controls (in total 25 libraries) simultaneously on the MiSeq and Ion Torrent PGM sequencers. FR2 EC-NGS primers, harbouring platform-specific sequencing adapters and sequencing barcodes, were used for the library preparation

based on one-step PCR. Five hundred ng of DNA per sample were used for library preparation.

The dilution experiments gave similar results (Figure 24) for both platforms, with a minimum sensitivity of 10^{-4} (as currently required by most treatment protocols using qPCR) for both tested assays. The correlation between MRD levels obtained by the two NGS platforms was good ($R^2=0.84$). In the low dilutions (10^{-1} and 10^{-3}) the correlation of the detected levels of target on both platforms was tight. Among 10^{-4} dilutions there were many outliers: three out of six targets were overestimated by Illumina and only one by Ion Torrent. This indicates that Ion Torrent better reflects the real dilutions. But on the other hand, in highly diluted samples (10^{-5}) MiSeq tends to have better sensitivity: all 10^{-5} dilutions were negative on Ion Torrent, whereas only 2 out of 6 targets were negative on MiSeq.

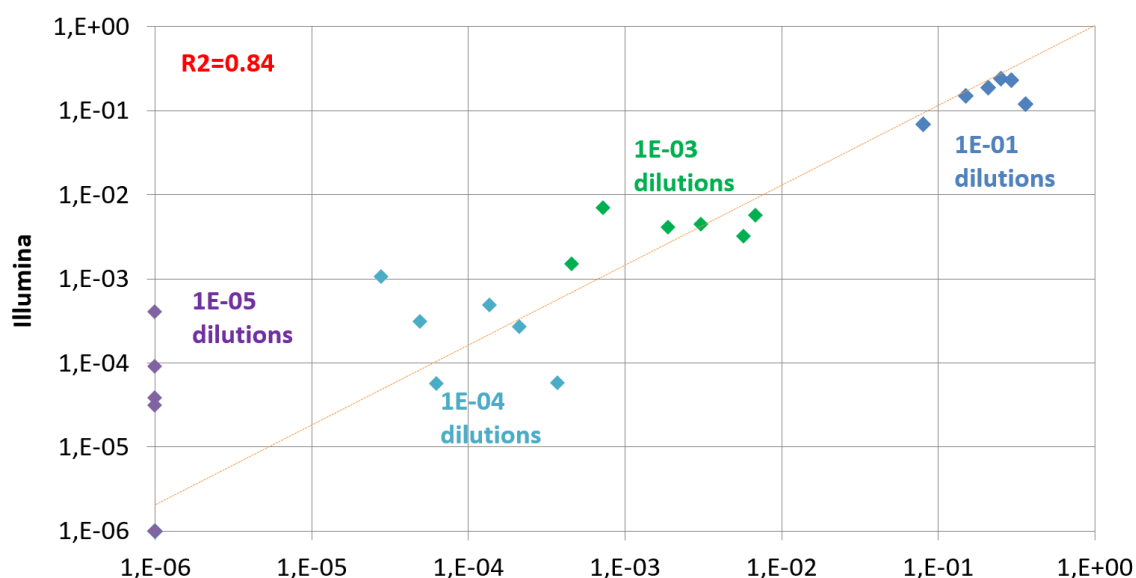


Figure 24: The graph showing the results of dilution experiment. The correlation of detected levels of target on MiSeq (Y-axis) and Ion Torrent (X-axis) are shown.

Figure 25 shows the examples of detected levels of target in the dilution series prepared from diagnostic samples of two BCP-ALL patients on MiSeq and Ion Torrent. Patient #1411 harboured two IGH targets.

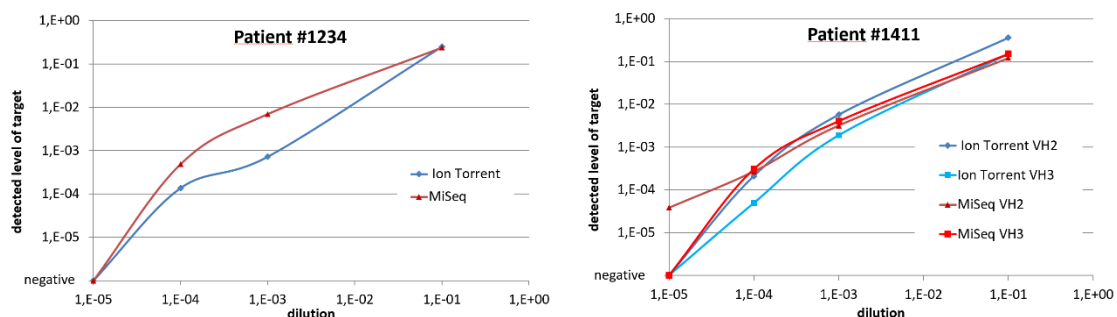


Figure 25: Detected levels of target in the dilution series prepared from diagnostic samples of two BCP-ALL patients (#1234 and #1411).

We compared the representation of the twenty most abundant V-J combinations within the control buffy coat libraries (4 replicates on MiSeq, 5 replicates on Ion Torrent). For sequencing on the MiSeq four independent sequencing libraries have been prepared. On Ion Torrent one library was sequenced five times. Ion Torrent data originating from a single library were more similar than the data from MiSeq (Figure 26). This indicates that the main source of differences between sequencing platforms is the library preparation and not the platform itself⁷⁸.

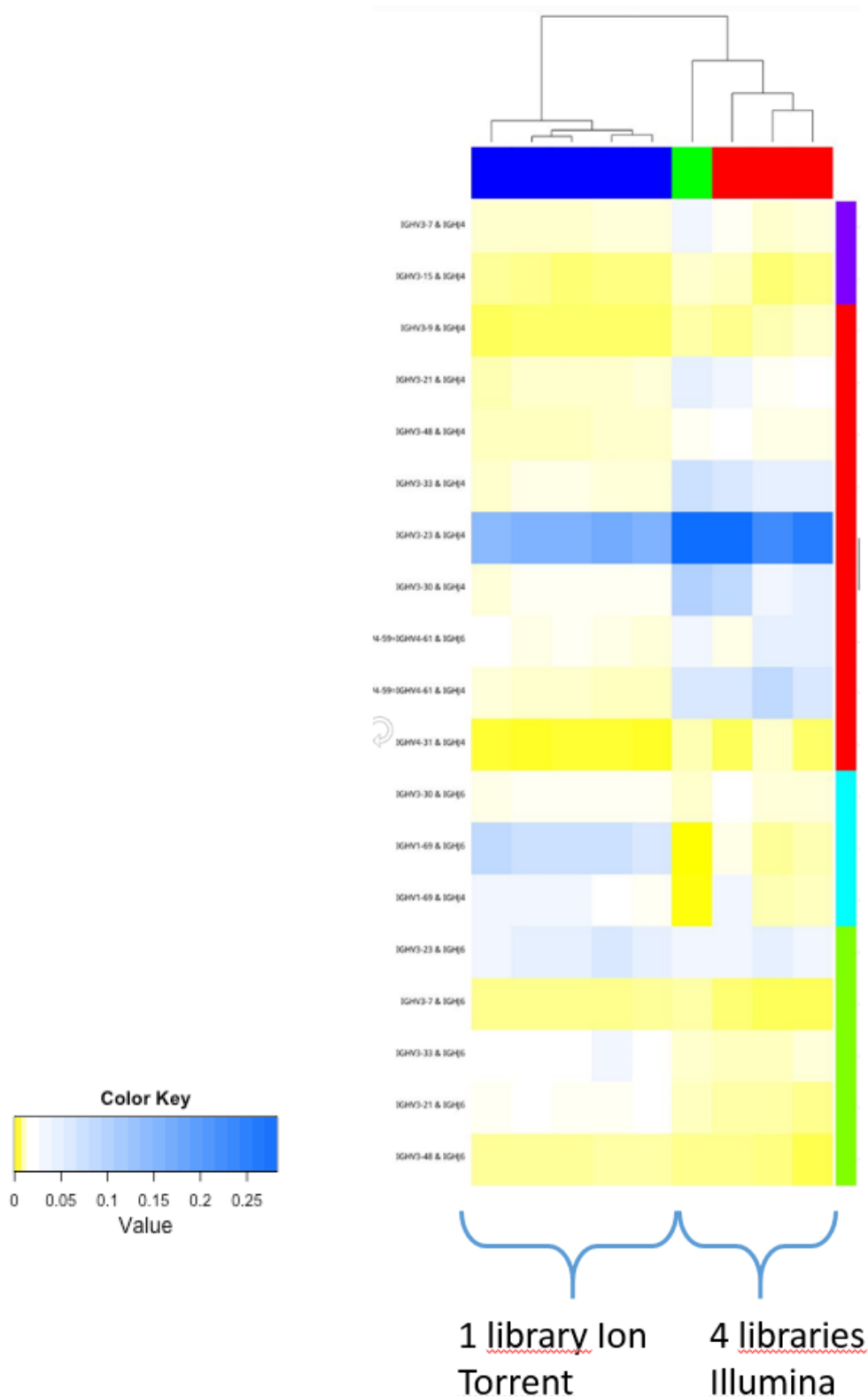


Figure 26: The heatmap representing the twenty most abundant V-J combinations within the control buffy coat (4 libraries on MiSeq, 1 library in 5 replicates on IonTorrent). The heatmap was created using the newly developed EC-NGS software Interrogate (to be published soon).

6. Discussion

One of the most important predictors of outcome in paediatric ALL is response to treatment which is represented by minimal residual disease level. MRD at days 33 and 78 is used on paediatric ALL treatment protocols to identify patients with low and high risk of relapse. The treatment intensity is then adapted according to the risk group classification.

The current gold standard method for MRD detection is qPCR for rearrangements of antigen receptors genes. It has been successfully used in paediatric ALL patients for almost two decades. Unfortunately this method is very laborious and time consuming, because it requires the optimization of target-specific assays for each patient.

Due to the long time required for the preparation of the specific qPCR assays it is impossible to use qPCR for online MRD monitoring at day 15. This is possible when using NGS, because NGS does not require patient-specific primers and assays – same universal consensus primers are used for all patients. The library preparation can be finished in a single day and template preparation with sequencing take another one and half day, therefore NGS-based MRD detection can be completed in less than one week.

NGS for MRD detection is not only faster, but also cheaper than qPCR-based MRD detection. The most costly part of the qPCR-based MRD detection is the patient-specific qPCR assay optimization (including the target identification, sequencing, patient-specific primers synthesis and PCR optimization). We calculated that qPCR is cheaper for MRD detection than NGS only when 6-7 time-points are measured. Moreover, only consumables are included in our calculation, but not the hands-on-time, which greatly dominates in qPCR.

We implemented the library preparation for Ion Torrent sequencers using multiplex fusion primer sets for NGS of the IgH (both VH-JH and DH-JH), TRG and TRD gene rearrangements and the two-round PCR. This approach is substantially cheaper than using the commercial kits for library preparation, which are based on the ligation of adaptors to the amplicons. In parallel we worked on the development and validation

of the platform-independent primer sets within the EC-NGS consortium. We sequenced the libraries created with the EC-NGS assays on the Ion Torrent PGM and the MiSeq sequencers and showed that both platforms provide comparable results. The EC-NGS primer sets are confidential at this point, but will soon be published.

We showed that the Ig/TR **target screening** based on NGS works very efficiently: the costs of both methods are comparable, but NGS is much faster than classical approach and can identify qPCR targets also in patients where Sanger sequencing fails. It is also capable of detecting targets in subclonal populations which are present at the lower frequencies. We plan to perform NGS target screening prospectively in all new ALL patients (using the primer sets from the EC-NGS consortium for all targets: IgH, TRB and TRD complete and incomplete, IgK and TRG) together with the classical method and to compare the results of both methods. After a testing period we plan to switch to NGS-based screening completely.

We proved that **MRD quantification** using NGS is a reliable tool for monitoring the treatment response. The sensitivity of both NGS and qPCR is limited by the number of analysed cells and is comparable when similar numbers of cells are analysed. The correlation of MRD levels detected by NGS and by qPCR was high. The vast majority of the discrepancies were found among samples with MRD levels below 10^{-4} which is below the theoretical sensitivity of both methods. Looking at the outcome of MRD-negative patients at day 33 we proved that NGS is more specific in relapse prediction at this time-point – NGS-MRD-negative group was slightly larger than qPCR-MRD-negative group and had a slightly better prognosis.

We showed that NGS is also more specific in regenerating **post-transplant** samples, where qPCR can detect **false positive results** due to the unspecific amplification of rearranged Ig/TR genes. Four out of 6 (67%) qPCR positive samples from patients with subsequent relapse vs. only 2 out of 34 (6%) qPCR positive samples from patients without relapse were positive also by NGS ($p=0.01$). We identified the sequences similar to all primers causing the unspecific amplification leading to the false positive MRD results also in the post-transplant samples from different patients, which confirms that the unspecific amplification is really an issue in these

samples. Besides avoiding the false positive results NGS can also prevent false negative MRD results caused by the clonal evolution.

Another advantage of NGS-based Ig/TR sequencing is that it provides the information about the entire antigen repertoire. At day 78 (week 12) from the therapy initiation the **repertoire diversity** can predict the risk of relapse. Patients with higher repertoire diversity at this time-point had longer relapse-free survival than patients with lower repertoire diversity. This probably reflects the better regenerating potential of bone marrow and thus the ability of the immune system to eliminate the residual leukemic cells. From our data it seems that the diversity of the immune repertoire is independent on MRD and its prognostic potential should be further explored.

7. Conclusions

NGS is a highly useful tool not only for MRD detection, but also for the assessment of the entire antigen repertoire. It can overcome some of the limitations of qPCR, namely the time-consuming optimisation of patient-specific assays and the risk of false positive/negative MRD results. Moreover, it provides additional information about the entire immunological background during the leukaemia treatment, while being at least price-comparable to qPCR.

Before the implementation of NGS-based MRD detection into the routine clinical practice, the intra- and inter-laboratory standardization of this method is necessary, as well as more extensive evaluation of its prognostic power. This is currently in progress (with contribution of our group) within the international EuroClonality-NGS consortium.

Articles Not Directly Related to the Topic of This Thesis

1) FRONKOVA E, KLOCPERK A, SVATON M, NOVAKOVA M, KOTROVA M, KAYSEROVA J, KALINA T, KESLOVA P, VOTAVA F, VINOHRADSKA H, FREIBERGER T, MEJSTIKOVA E, TRKA J and SEDIVA A. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. PLoS ONE. 2014. Vol. 9, no. 12. DOI 10.1371/journal.pone.0114514. IF:3.23

During the process of V-(D)-J recombination parts of the cleaved DNA are circularized and stay non-degraded in the cells. In this work we performed qPCR-based detection of T-cell recombination excision circles (TREC) and kappa-deleting element recombination circles (KREC) in 13 new-borns with DiGeorge syndrome, as well as in 56 healthy new-borns. TREC/KREC levels reflect the numbers of newly developed T-/B-lymphocytes. We showed that the screening based on the TREC/KREC levels detection does not identify patients with DiGeorge syndrome at birth, because these patients have TREC/KREC levels in the normal range.

2) DYTRYCH P, KROL P, KOTROVA M, KUZILKOVA D, HUBACEK P, KROL L, KATRA R, HRUSAK O, KABELKA Z, DOLEZALOVA P, KALINA T and FRONKOVA E. Polyclonal, newly derived T cells with low expression of inhibitory molecule PD-1 in tonsils define the phenotype of lymphocytes in children with Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome. Molecular immunology. 2015. Vol. 65, no. 1, p. 139–47. DOI 10.1016/j.molimm.2015.01.004. IF: 2.97

We investigated the lymphocyte subsets in tonsils and peripheral blood samples from 10 children with Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome and also 10 children with obstructive sleep apnoea who served as healthy controls. We observed approximately 2-fold increase of CD8+ T cell proportion in the PFAPA tonsils compared to controls. The PFAPA tonsils had slightly elevated numbers of $\gamma\delta$ T-lymphocytes and the next generation sequencing of TRG rearrangements in 4 PFAPA tonsils detected a highly represented clone CALWEVYYKKL (which was previously detected in a patient with periodic fever) in one of the patients. No clonal expansion was present in the samples from PFAPA tonsils.

3) BOUBLIKOVA, L, BAKARDJIEVA-MIHAYLOVA, V, SKVAROVA KRAMARZOVA, K, KUZILKOVA, D, DOBIASOVA, A, FISER, K, STUCHLY, J, KOTROVA, M, BUCHLER, T, DUSEK, P, GREGA, M, ROSOVA, B, VERNEROVA, Z, KLEZL, P, PESL, M, ZACHOVAL, R, KROLUPPER, M, KUBECOVA, M, STAHALOVA, V, ABRAHAMOVA, J, BABJUK, M, KODET, R and TRKA, J. Wilms tumor gene 1 (WT1), TP53, RAS/BRAF and KIT aberrations in testicular germ cell tumors. *Cancer letters*. 2016. Vol. 376, no. 2, p. 367–376. DOI 10.1016/j.canlet.2016.04.016. IF: 5.62

We used amplicon next generation sequencing for the detection of mutation in 26 testicular germ cell tumours (TGCT) samples. Using this approach we detected ATM variants in the largest number of TGCT samples (at least one single nucleotide variant found in 42% of samples), followed by variants in BRAF (27%), WT1 (23%) and RAS genes (19%). Quantitative PCR for WT1 expression showed that WT1 is under-expressed in TGCT in comparison to non-malignant testicular tissue. Based on this findings we concluded that WT1 is an important factor involved in TGCT pathogenesis.

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Attachments

To the editor:

The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL

Minimal residual disease (MRD) monitoring via antigen receptor quantitative polymerase chain reaction (qPCR) is an important predictor of outcome in childhood acute lymphoblastic leukemia (ALL), is rigorously standardized within the EuroMRD consortium and has a greater sensitivity than flow cytometry (FC), which has been used in other trials.¹ However, qPCR is laborious, expensive, and time consuming because of the development of patient-specific assays. MRD detection based on next-generation sequencing (NGS) of antigen receptor gene rearrangements is a promising tool that permits sequencing of large numbers of rearranged V-(D)-J segments and thus provides the picture of not only residual leukemia but also the normal immune repertoire of respective cells.²⁻⁵

To date, no correlation study on larger number of samples has been performed to determine how the discrepancies between NGS and qPCR data would affect clinical data. Moreover, there are no data investigating the impact of normal B-cell compartment reconstitution after induction treatment in ALL on prognosis. We developed a simple, cost effective, and easily adoptable approach using 2-round

PCR amplifying virtually all immunoglobulin heavy chain (IgH) rearrangements⁶ and NGS on Ion Torrent/Proton sequencers (supplemental Methods; available on the *Blood* Web site). We compared the NGS-MRD results with current techniques⁷ in patients treated by Berlin-Frankfurt-Munster (BFM)-based protocol and investigated the changes in the treatment stratification using the new method.

Altogether, we sequenced 210 samples from 76 patients (62 × day 15, 73 × day 33, 75 × day 78) with a median coverage of 729 842 reads per sample. The overall correlation of NGS- and qPCR-MRD in all samples was satisfactory ($R^2 = 0.72$; Figure 1A). A total of 32 samples (15%) were positive by NGS/negative by qPCR or vice versa, causing a shift in BFM risk stratification in 25 patients (33%), mainly between standard-risk (SR) and intermediate-risk (IR) groups in the low-positive patients. Five patients would be reassigned from the IR to the slow early responder (SER) group (4 of them relapsed), and 2 patients from SER to IR (1 of them relapsed).

NGS-MRD positivity at day 33 provided a more accurate prediction of relapse than qPCR-MRD positivity (Figure 1B-C) (5-year

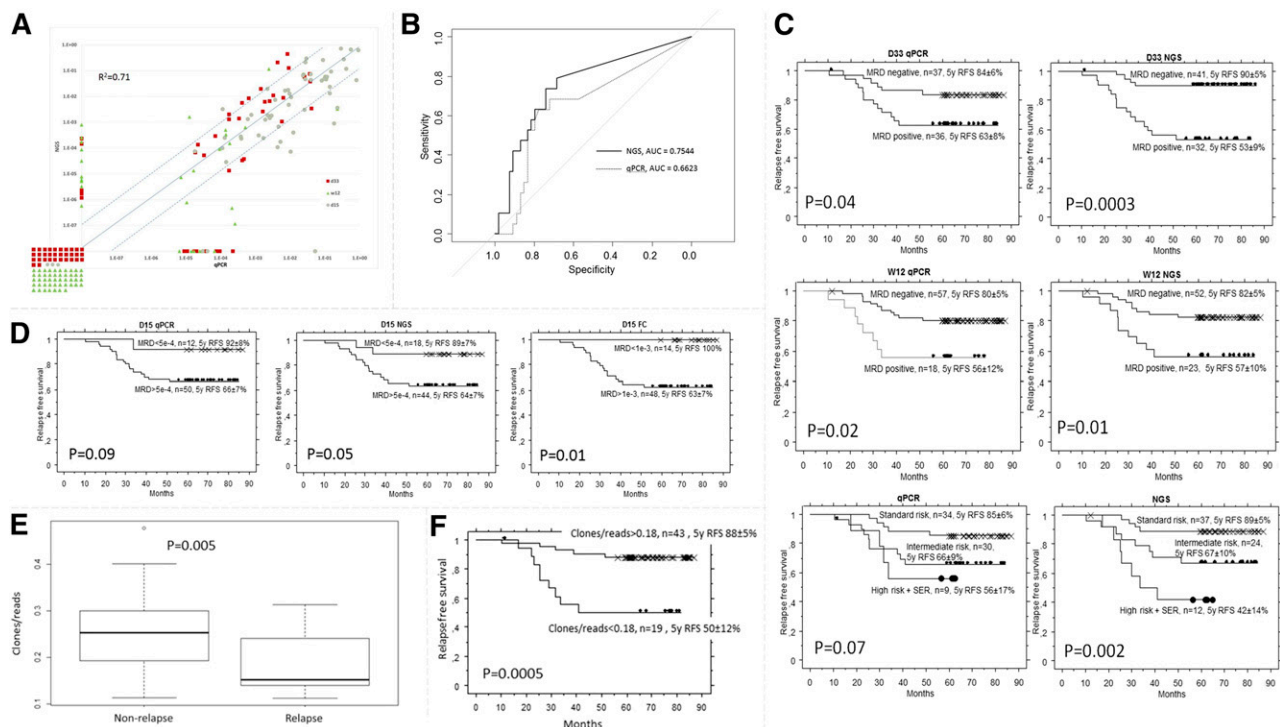


Figure 1. The comparison of MRD as measured by qPCR and NGS, the prognostic significance of NGS, qPCR and FC at various treatment time points and the impact of IgH repertoire diversity on outcome. (A) The comparison of MRD as measured by qPCR and NGS. MRD at different time points (days 15, 33, and 78) by qPCR (x-axis) and NGS (y-axis). (B) Receiver operating characteristic curves and corresponding areas under the curve (AUC) statistics for day 33 NGS-MRD and day 33 qPCR-MRD prediction of relapse show better specificity of NGS over qPCR. (C) Prognostic significance of MRD at days 33 and 78 assessed simultaneously by qPCR and NGS. Kaplan-Meier survival plots show relapse-free survival (RFS) of patients with ALL based on MRD at day 33, MRD at day 78, and assigned via BFM risk group stratification into 3 risk groups using combined day 33 and day 78 MRD levels. At day 33, NGS defined a slightly larger (41 vs 37 patients, not significant) group of patients with negative MRD than qPCR, who had a similarly good prognosis as those defined by qPCR, even though 22% of patients in the NGS negative group were qPCR positive. The superiority of NGS over qPCR at day 33 was confirmed by deviance analysis of a multivariate Cox model, which showed a significant improvement of NGS prediction over qPCR ($P = .003$). As shown in the supplemental Methods, the total cohort was enriched for patients with relapse, which causes artificially low RFS in respective subgroups. (D) Prognostic significance of MRD at day 15 assessed by qPCR, NGS, and FC. Kaplan-Meier survival plots show RFS of patients with ALL based on MRD at day 15. (E) The impact of IgH repertoire diversity on outcome. Normalized IgH repertoire diversity expressed as number of clones/number of reads at day 78 for patients with and without relapse. The samples with NGS-MRD higher than 10^{-4} were excluded from the analysis. (F) RFS according to normalized IgH repertoire diversity at day 78. Kaplan-Meier survival RFS of patients with ALL based on repertoire diversity at day 78. Patients with clonal diversity (number of clones/number of reads) of less than 0.18 at day 78 had a 5-year RFS = $50 \pm 12\%$ compared with $88 \pm 5\%$ in other patients ($P = .0005$). Interestingly, of 9 patients who relapsed in the group with low diversity, 4 were stratified into the SR group according to current qPCR-based stratification (combined day 33/day 78). d15, day 15; d33, day 33; d78, day 78.

RFS NGS-negative/positive: $90 \pm 5\%$ vs $53 \pm 9\%$, $P = .0003$; PCR-negative/positive: $84 \pm 6\%$ vs $63 \pm 8\%$, $P = .04$). Deviance analysis of a multivariate Cox model showed significant improvement of NGS prediction over qPCR ($P = .003$). At day 78, the predictive value of NGS was almost identical to qPCR (Figure 1C). Combined day 33 and 78 information used for defining SR, IR, and high risk (+ SER) groups on BFM trials gave slightly better results than qPCR (Figure 1C). In consensus with published reports,^{8,9} both 8-color FC and qPCR defined a subgroup of patients with low MRD as early as day 15, who had a superior prognosis. The NGS approach identified a similarly large group of low-risk patients with excellent outcome (Figure 1D).

NGS data provide information not only about MRD but also about the rest of the B-lymphoid repertoire. We assessed clonal heterogeneity of the IgH repertoire in investigated samples, using the Vidjil algorithm¹⁰ (supplemental Methods). Interestingly, the patients with relapse had significantly lower IgH repertoire diversity at days 33 and 78. Even after removal of patients with NGS-MRD higher than 10^{-4} , the patients with lower clonal diversity at day 78 had significantly worse 5-year RFS than other patients ($P = .0005$; Figure 1F).

In conclusion, the NGS risk group assignment is partly different from current approaches. This is mostly because of discrepancies in low-positive samples in SR/IR groups, but with no negative impact on final outcome. The NGS even provided a more precise prediction of relapse than qPCR at day 33 in our limited cohort. However, a prospective validation study comparing both methods will be needed to definitely accept NGS as the replacement for qPCR. A redefinition of stratification criteria for childhood ALL will be only the final step of a complex process that has just started. The main challenge in NGS-MRD methodology is standardization aiming at highly reproducible results between different centers, as it was achieved previously within the Euro-MRD group for qPCR. A European network, the EuroClonality-NGS Consortium, has been formed to standardize the workflow of analytics, preanalytics, and bioinformatics. Before this is accomplished, the methodology described in our study can serve as a broadly accessible and relatively inexpensive noncommercial solution for centers that do not perform antigen receptor qPCR but want to start using benefits of MRD monitoring in their clinical setting.

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The online version of this article contains a data supplement.

Acknowledgments: This work was supported by grants from the Internal Grant Agency of the Czech Ministry of Health (IGA MZCR NT14343), the Grant Agency of the Czech Republic (GACR, project of Centre of Excellence No. P302/12/G101)

(J.T.), the Charles University Grant Agency (GAUK 394214) (M.K.), and the University Hospital Motol, Prague, Czech Republic (00064203) (K.M.).

Contribution: M.K. prepared the sequencing libraries, performed the sequencing runs, and performed the data analysis; K.M. prepared the sequencing libraries and performed the sequencing runs; V.B.-M. performed the sequencing runs; M.N. and E.M. performed the FC analysis; K.F. and J. Stuchly developed the bioinformatic tools for MRD tracking; M.G. and M.S. performed the repertoire analysis; C.P. and M.B. developed the protocol for library preparation and interpreted the data; M.F. developed the protocol for library preparation; J. Stary and J.T. planned the study and interpreted the data; and E.F. planned the study, interpreted the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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DOI 10.1182/blood-2015-07-655159

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2015 126: 1045-1047

doi:10.1182/blood-2015-07-655159

The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL

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Supplementary methods, results, figures and tables

The Predictive Strength of Next Generation Sequencing MRD Detection for Relapse Compared with Current Methods in Childhood ALL

Michaela Kotrova, Katerina Muzikova, Ester Mejstrikova, Michaela Novakova, Violeta Bakardjieva-Mihaylova, Karel Fiser, Jan Stuchly, Mathieu Giraud, Mikaël Salson, Christiane Pott, Monika Brüggemann, Marc Füllgrabe, Jan Sary, Jan Trka, Eva Fronkova

Supplementary methods

Patients, samples and treatment protocol

In total, 76 patients with B-cell precursor ALL were investigated. Sixty-two patients treated according to the AIEOP-BFM-ALL 2000 protocol^{1,2} were included based on the availability of 1-2 IGH-VH-JH qPCR targets with a minimal sensitivity of 10^{-4} , according to the EuroMRD criteria³. To better describe MRD kinetics in relapsed cases, which are rare in pediatric ALL, 14 consecutive patients with available qPCR IGH targets who relapsed from previous (ALLIC-BFM 2002⁴) and current (AIEOP-BFM-ALL 2009) frontline treatment protocols were added to the cohort. Patient characteristics are shown in the Supplementary Table 1.

Stratification into risk groups was assigned based on MRD at days 33 and 78. Current treatment protocol of the AIEOP/BFM Group (ALL-BFM 2009) requires just one sensitive marker per patient for the treatment stratification. This is based on the results of the previous protocol (AIEOP-BFM 2000), which required 2 markers for MRD stratification¹. Accordingly, our own results showed that one marker was sufficient in BFM induction treatment⁴. The patients were stratified as MRD standard risk (PCR-SR) if MRD was negative at both days 33 and 78, with a sensitivity of at least 10^{-4} . Patients with MRD higher or equal to 10^{-3} at day 78 were treated in the high risk arm of the protocol (PCR-HR), independent of the sensitivity of the PCR. A new subgroup of HR called slow early responders (SER) has been defined based on previous treatment results¹ as having MRD higher or equal to 10^{-3} at day 33 and positive at day 78. Patients with other MRD values were considered to be of intermediate risk (PCR-IR). Patients with poor response to the initial treatment phase, consisting of one week of prednisone with one methothrexate (prednisone poor response, PPR; ≥ 1000 leukemic blasts per microliter in the peripheral blood on day 8), or who failed to achieve remission (i.e., with $> 5\%$ leukemic blasts in the bone marrow (BM) on day 33, or

persistent extramedullary disease) after induction phase IA or positivity for MLL/AF4 fusion transcripts, were treated in the HR arm, irrespective of their MRD results¹. The treatment stratification in the AIEOP-BFM-ALL 2009 study was similar to the AIEOP-BFM-ALL 2000 study; one Ig/TCR target with a sensitivity of at least 10^{-4} was required. Patients with FC MRD higher than 10% at day 15 were stratified to HR group, irrespective of the qPCR results at day 33 or day 78. The treatment stratification in the ALLIC-BFM 2002 protocol was not based on MRD and was performed using other criteria as described previously.

The additional 14 patients with subsequent relapse were treated according to other BFM-backbone protocols (10 with ALL-IC BFM 2002, two with AIEOP-BFM-ALL 2009 and two with ALL Interim BFM 2007).

Sample processing and MRD analysis using qPCR/flow cytometry

Mononuclear cells from 210 samples from 76 patients (62×day 15, 73×day 33, 75×day 78) were isolated using the Ficoll-Paque density centrifugation method (Pharmacia, Uppsala, Sweden). The DNA was isolated using QIAamp DNA Mini/Micro Kits (Qiagen). Flow cytometry evaluation of lymphocyte subpopulations (mainly the percentage of CD19^{pos} cells) was performed in parallel to molecular genetic testing, as described previously⁵, in all follow-up samples to facilitate the optimization process. However, they were not used for the correction of the NGS-MRD results.

MRD analysis using qPCR quantification of clonal IGH rearrangement consisted of identifying clonal IGH targets⁶, Sanger sequencing of junctional V-D-J regions, design of patient-specific primers, and optimization of qPCR assays.^{4,7} The results were interpreted according to EuroMRD guidelines⁵ after normalization to the level of the control gene, albumin.⁸

MRD analysis using amplicon NGS

Each sequencing library was created from 450 ng (the equivalent of 70,000 DNA copies) of bone marrow DNA in all follow-up samples and 50 ng of Human Genomic DNA (Roche) using 2 round PCR. In the first PCR round, rearranged IGH genes were amplified using the IGH FR3 Biomed-2 multiplex system with seven forward primers for each IGH gene family and one reverse primer common for all JH segments⁹. The Biomed-2 primers were modified by adding universal M13 adaptors at the 5' end (Supplementary Table 2). All primers used for library preparation were diluted in salmon sperm (Sigma-Aldrich), instead of water, to improve their

stability. The PCR protocol is described in detail in Supplementary Table 3. The 1st round PCR products were purified by Agencourt AMPure XP beads (Beckman Coulter) (Beads:DNA ratio 1.5) and diluted 50 times prior to the 2nd round. In the second PCR round, Ion Torrent adapters and multiplex identifiers, enabling the parallel sequencing of up to 50 samples and also containing M13 adaptors, were added to the 1st round products (Supplementary Table 2). The examples of resulting sequencing libraries are shown in Supplementary Figure 1.

The quality and concentration of libraries were analyzed with the Agilent High Sensitivity DNA kit. The libraries were diluted to 26 pmol/l (PGM) or 11 pmol/l (Proton) and pooled at equimolar concentrations. Sequencing was performed on Ion Torrent PGM/Ion Proton machines, using 200 bp chemistry as follows: Ion PGM™ Template OT2 200 Kit + Ion PGM™ Sequencing 200 Kit v2 or Ion PI™ Template OT2 200 Kit v2 + Ion PI™ Sequencing 200 Kit v2.

Bioinformatic analysis of NGS data.

The basic quality filtering of sequencing data and generating of separate fastq¹⁰ files for each respective barcode were done by the Torrent Suite™ Software (4.2). Fragments shorter than 100 bp were filtered out using the R package. For each patient, clonal IGH rearrangement(s) obtained by Sanger sequencing from diagnostic BM DNA samples were manually analyzed and the CDR3 region with V-D-J borders was identified. The Ion Torrent/Proton data contained substantial number of imprecisions in homopolymer stretches. As such, 15 bp-long markers containing a maximum of 10 bp from the germline V, D or J segments were defined in a sliding window with a 2 bp overlap (Supplementary Figure 2). For each patient, the fastq results files were searched for all selected marker sequences and the reads containing selected strings were listed in a txt file using grep utility search. The resulting file was visually checked for large discrepancies, which can be caused by long homopolymer stretches or by ongoing rearrangements with V-V replacement with a common D-J stem. In cases of large discrepancies, such markers were excluded from further analysis. Subsequently, MRD was set as the average number of marker sequences per total number of reads x 0.9 (considering 10% of sequences from added polyclonal DNA). If two clonal IGH targets were available, both of them were evaluated and a higher value was used for MRD.

Bioinformatics estimation of clonal heterogeneity

The sequencing data were processed with the open-source Vidjil software, version 2015.04,

using the IGH preset (www.vidjil.org). Reads are gathered in a same clone when they share at the nucleotide level an exact 60 bp window centered on the CDR3. The position of this window is detected by considering the conserved V and J segments closest to the junction¹¹. V and J genes databases were taken from the IMGT/GENE-DB database, germline version 201513-0¹². Sequencing errors may lead to the detection of extraneous clones, but their number will be related to the number of actual clones. When all the data was sequenced on a same sequencer, the clones/reads ratio can thus be considered as an indicator of the diversity of the repertoire. A large clone/reads ratio is correlated to a large number of clones gathering a few proportion of reads (data not shown).

Statistical analysis

All statistical analyses were performed using StatView® version 5.0 (StatView® Software, Cary, NC). The distribution of variables between groups was assessed using Fisher's exact test. Relapse-free survival was described using Cox proportional hazards model.

Supplementary results

For the validation of the assay, we sequenced 10^{-1} to 10^{-5} dilutions of two diagnostic samples with different IGH rearrangements in multiplicates. The results showed that the assay gave reproducible quantitative results up to 5×10^{-4} dilution and reached a sensitivity of 10^{-5} in 2 of 4 replicates in both patients (Supplementary Figure 3).

To discern whether the NGS-negative/qPCR positive results were caused by a lower sensitivity of NGS caused by underperformance of respective V-J primer combinations, or due to possible false positivity by qPCR, we tested the individual sensitivity of NGS for 7 patients with NGS-negative/qPCR-positive PCR result. We performed sequencing of 10^{-4} and 10^{-5} dilutions of diagnostic samples in these patients (data not shown). In 5 of 7 samples, the 10^{-5} dilutions gave positive reads in NGS experiment, and in the remaining two the 10^{-4} dilution was detected, which shows that the negative results were not caused by insufficient sensitivity of NGS in these patients.

When considering the treatment stratification timepoints separately, the NGS approach detected significantly lower MRD than qPCR at day 33 in patients who did not relapse. Specifically, when analyzing only double-positive samples, 13 of 25 patients who did not relapse had NGS-MRD at day 33 lower than qPCR vs. 2 of 15 patients with relapse (Fisher

exact test, $p=0.01$, Supplementary Table 4, Supplementary Figure 4).

This trend was mostly caused by qPCR-low-positive/NGS-negative d33 samples in patients without relapse, suggesting a better specificity of NGS for relapse prediction in such samples.

Supplementary Table 1: Patient characteristics

	Study cohort				Interim AIEOP-BFM-ALL 2000				Significance
Age	<6years		>6years		<6years		>6years		$p=0.17$
	41	53.95%	35	46.05%	124	62.94%	73	37.06%	
Gender	Male		Female		Male		Female		$p=0.73$
	48	63.16%	28	36.84%	120	60.91%	77	39.09%	
Leukocytes at diagnosis	<20,000		>20,000		<20,000		>20,000		$p=0.88$
	51	67.11%	25	32.89%	129	66.15%	66	33.85%	
MRD Risk group	SR	IR	SER	HR	SR	IR	SER	HR	$p=0.19$
	35	29	9	3	85	78	8	8	
	46.05%	38.16%	11.84%	3.95%	47.49%	43.58%	4.47%	4.47%	

Supplementary Table 2: Reaction and cycling conditions for sequencing library preparation.

1 st round	2 nd round
Reaction conditions Buffer: ABI PCR Buffer II 10x 25 μ l final volume 450 ng DNA 2,5 pmol of each primer dNTP: 5nmol MgCl ₂ : 37,5nmol Taq enzyme: 1U Cycling conditions Preactivation 94°C 10min Denaturation 94°C 1min Annealing 63°C 1min Extension 72°C 30s Final extension 72°C 30min Number of cycles: 25 Hold 4°C	Reaction conditions Buffer: FastStart High Fidelity Reaction Buffer, 10x concentrated, with 18mM MgCl ₂ 25 μ l final volume 1 μ l 50x diluted 1st round product, purified with Agencourt AMPure Beads 5 pmol of each primer dNTP: 5nmol FastStart High fidelity polymerase: 2,5U Cycling conditions Preactivation 95°C 2min Denaturation 95°C 30s Annealing 63°C 30s Extension 72°C 30s Final extension 72°C 5min Number of cycles: 25 Hold 4°C

Supplementary Table 3: Primers for library preparation^a.

1 st PCR round primers (5'-3'):		
	5'	3'
forward	VH1-FR3- M13F	GTAAACGACGGCCAGTGGAGCTGAGCAGCCTGAGATCTGA
	VH2-FR3- M13F	GTAAACGACGGCCAGCAATGACCAACATGGACCCTGTGGA
	VH3-FR3- M13F	GTAAACGACGGCCAGTCTGCAAATGAACAGCCTGAGAGCC
	VH4-FR3- M13F	GTAAACGACGGCCAGGAGCTCTGTGACCGCCGCGACACG
	VH5-FR3-	GTAAACGACGGCCAGCAGCACCGCCTACCTGCAGTGGAGC

	M13F	
	VH6-FR3-M13F	GTAAACGACGGCCAGGTTCTCCCTGCAGCTGAACTCTGTG
	VH7-FR3-M13F	GTAAACGACGGCCAGAGCCTAAAGGCTGAGGACAC
reverse	JHc-M13R	CAGGAACAGCTATGACCTTACCTGAGGAGACGGTGACC
	2nd PCR round primers(5'-3'):	
		5' 3'
forward	A-MID1-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACGATGTAAACGACGGCCAG
	A-MID2-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACGATGTAAACGACGGCCAG
	A-MID3-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGATTTCGATGTAAACGACGGCCAG
	A-MID4-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGATGTAAACGACGGCCAG
	A-MID5-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGATGTAAACGACGGCCAG
	A-MID6-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCGATGTAAACGACGGCCAG
	A-MID7-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTTCGATGTAAACGACGGCCAG
	A-MID8-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACGATGTAAACGACGGCCAG
	A-MID9-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACGATGTAAACGACGGCCAG
	A-MID10-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACGATGTAAACGACGGCCAG
	A-MID11-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCGATGTAAACGACGGCCAG
	A-MID12-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCGATGTAAACGACGGCCAG
	A-MID13-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACGATGTAAACGACGGCCAG
	A-MID14-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTTCGATGTAAACGACGGCCAG
	A-MID15-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCGATGTAAACGACGGCCAG
	A-MID16-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACGATGTAAACGACGGCCAG
	A-MID17-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTTCGTCGATGTAAACGACGGCCAG
	A-MID18-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCGATGTAAACGACGGCCAG
	A-MID19-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACGATGTAAACGACGGCCAG
	A-MID20-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCGATGTAAACGACGGCCAG
	A-MID21-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACGATGTAAACGACGGCCAG
	A-MID22-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCGATGTAAACGACGGCCAG
	A-MID23-M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACGATGTAAACGACGGCCAG

	M13F	
	A-MID24- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTGATGTAAACGACGGCCAG
	A-MID25- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACGATGTAAACGACGGCCAG
	A-MID26- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCGATGTAAACGACGGCCAG
	A-MID27- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCGATGTAAACGACGGCCAG
	A-MID28- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCGATGTAAACGACGGCCAG
	A-MID29- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCGATGTAAACGACGGCCAG
	A-MID30- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTATCGATGTAAACGACGGCCAG
	A-MID31- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCGATGTAAACGACGGCCAG
	A-MID32- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACGATGTAAACGACGGCCAG
	A-MID33- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTCATTGAACGATGTAAACGACGGCCAG
	A-MID34- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCATCGTTCGATGTAAACGACGGCCAG
	A-MID35- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCCATTGTCGATGTAAACGACGGCCAG
	A-MID36- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGAATCGTCGATGTAAACGACGGCCAG
	A-MID37- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGAGAATGTCGATGTAAACGACGGCCAG
	A-MID38- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGGACGGACGATGTAAACGACGGCCAG
	A-MID39- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAATCGGCGATGTAAACGACGGCCAG
	A-MID40- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACATAATCGATGTAAACGACGGCCAG
	A-MID41- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCACTTCGCGATGTAAACGACGGCCAG
	A-MID42- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACGAATCGATGTAAACGACGGCCAG
	A-MID43- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGACACCGCGATGTAAACGACGGCCAG
	A-MID44- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGGCCAGCGATGTAAACGACGGCCAG
	A-MID45- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGCTTCCTCGATGTAAACGACGGCCAG
	A-MID46- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGTCCGAACGATGTAAACGACGGCCAG

	A-MID47- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGCAACCACGATGTAAACGACGGCCAG
	A-MID48- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTAAGAGACGATGTAAACGACGGCCAG
	A-MID49- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTAACATAACGATGTAAACGACGGCCAG
	A-MID50- M13F	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGGACAATGGCGATGTAAACGACGGCCAG
reverse	trP1-M13R	CCTCTCTATGGGCAGTCGGTGATCAGGAAACAGCTATGAC

^aprimers for 1st round PCR (adapted from van Dongen et al, Leukemia 2003).

Supplementary Table 4: Comparison of NGS and qPCR-based MRD at day 33 in relapsed vs. non-relapsed patients.

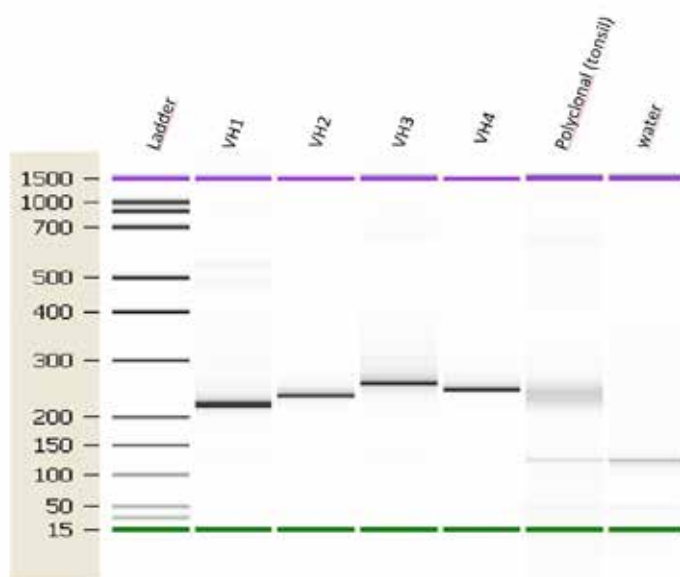
Day 33 without double negative samples

	NGS>qPCR	NGS≤qPCR
relapse	13	2
non-relapse	12	13

p=0.01

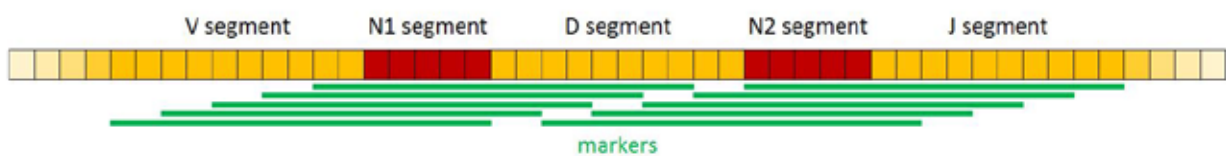
Supplementary Figure 1: An example of the Agilent electropherogram of the final sequencing libraries.

Diagnostic DNA samples from the bone marrow of 4 patients with four different monoclonal IGH rearrangements and a polyclonal control from a healthy donor are shown.

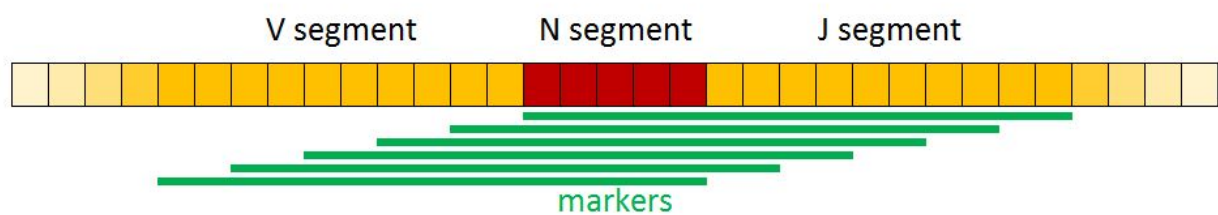


Supplementary Figure 2: The strategy for MRD analysis. Multiple 15 bp long MRD markers in a sliding window with a 2 bp overlap were defined based on the IGH sequence from the diagnosis of each patient for A) targets with 2 N segments and B) targets with one N segment (e.g., D segment of at least 5 bp not identified). The first marker starts -10 bp in the V segment and the last one ends at +10 bp in the J segment. If a D segment larger or equal to 10 nt was identified, the last marker for N1 segment ends -10 bp in the D segment and the first marker for N2 segment starts -10 bp in the D segment.

A

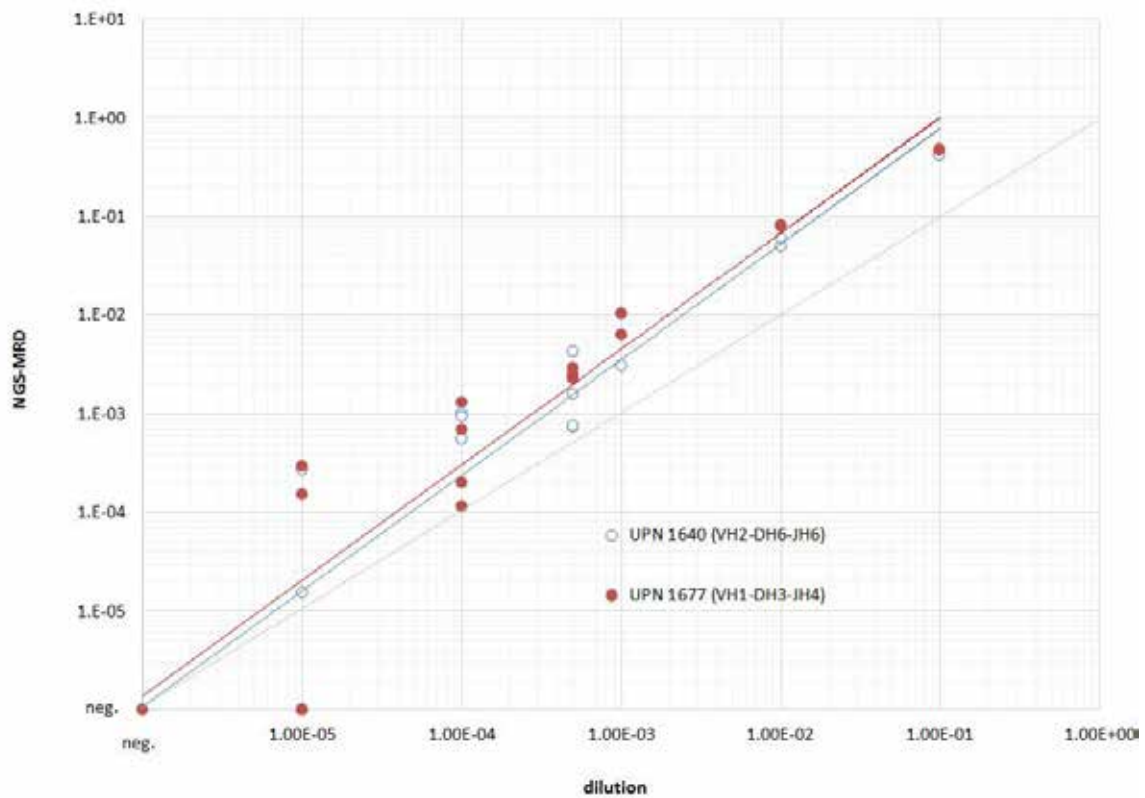


B

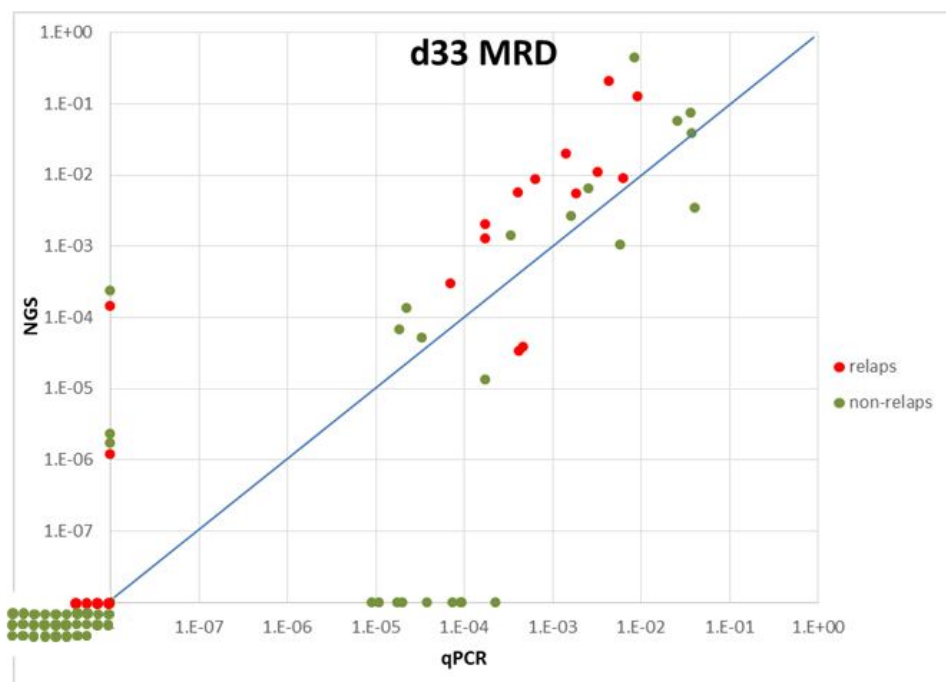


Supplementary Figure 3: A dilution experiment exploring the sensitivity and dynamic range of the NGS method.

Diagnostic leukemic DNAs of two patients with ALL, each having one clonal IGH rearrangement (VH4-JH6, full circles, and VH3-JH3, empty circles), were logarithmically diluted into polyclonal DNA of healthy donors. Each dilution step was processed by NGS, with a median coverage of 401,802 reads, the 10^{-2} and 10^{-3} steps in duplicates, the 5×10^4 , 10^{-4} and 10^{-5} steps in quadruplicate. One polyclonal sample was added as a negative control. The number of reads with diagnostic clonal IGH rearrangements in all reads of expected size is shown on the y-axis. *ALL*, acute lymphoblastic leukemia; *IGH*, immunoglobulin heavy chain, *NGS*, next generation sequencing; *qPCR*, quantitative polymerase chain reaction.



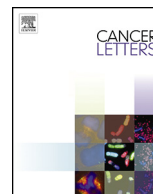
Supplementary Figure 4: The comparison of MRD as measured by qPCR (x-axis) and NGS (y-axis) in patients who underwent relapse vs. non-relapsed patients at day 33.



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Original Articles

Wilms tumor gene 1 (WT1), TP53, RAS/BRAF and KIT aberrations in testicular germ cell tumors



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ARTICLE INFO

Article history:

Received 22 December 2015

Received in revised form 4 April 2016

Accepted 8 April 2016

Keywords:

Wilms tumor gene 1 (WT1)

TP53

KIT

Testicular germ cell tumors

Next generation sequencing

ABSTRACT

Purpose: Wilms tumor gene 1 (WT1), a zinc-finger transcription factor essential for testis development and function, along with other genes, was investigated for their role in the pathogenesis of testicular germ cell tumors (TGCT).

Methods: In total, 284 TGCT and 100 control samples were investigated, including qPCR for WT1 expression and BRAF mutation, p53 immunohistochemistry detection, and massively parallel amplicon sequencing.

Results: WT1 was significantly ($p < 0.0001$) under-expressed in TGCT, with an increased ratio of exon 5-lacking isoforms, reaching low levels in chemo-naïve relapsed TGCT patients vs. high levels in chemotherapy-pretreated relapsed patients. BRAF V600E mutation was identified in 1% of patients only. p53 protein was lowly expressed in TGCT metastases compared to the matched primary tumors. Of 9 selected TGCT-linked genes, RAS/BRAF and WT1 mutations were frequent while significant TP53 and KIT variants were not detected ($p = 0.0003$).

Conclusions: WT1 has been identified as a novel factor involved in TGCT pathogenesis, with a potential prognostic impact. Distinct biologic nature of the two types of relapses occurring in TGCT has been demonstrated. Differential mutation rate of the key TGCT-related genes has been documented.

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Introduction

Testicular germ cell tumors (TGCT), the most common solid tumors and cause of cancer-related mortality in young men aged 18–35 years, are malignancies with unique biologic and molecular features that determine the clinical course of the disease and overall excellent treatment results [1]. However, the progress in understanding the molecular basis of this disease seems to be slower than in other tumors; in particular, no molecular prognostic or

predictive factors or targeted therapy have been implemented into the treatment protocols so far, and the management and outcomes of patients with TGCT have not changed for decades [2,3]. In patients with early stage TGCT, it is essential to introduce novel indicators of relapse risk that would improve patient stratification for adjuvant treatment (mostly chemotherapy) thus decreasing the cytotoxic load and the occurrence of late side-effects in long-term survivors. For patients with advanced TGCT, we need an improved treatment strategy and efficient approach to those with developed cisplatin-resistance, which is the principal cause of treatment failure. Further research in TGCT biology and exploration of factors with potential clinical impact are therefore essential.

Wilms tumor gene 1 (WT1) on chromosome 11p13 encodes a zinc-finger transcription factor with complex regulatory function. It is

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crucial for normal development of the urogenital system, all mesenchymal tissues and maintenance of epithelial-mesenchymal balance. *WT1* alterations may lead to disturbed mesenchymal to epithelial transition (MET), resulting in aberrant proliferation associated with malignant tumors as well as non-malignant diseases (e.g. glomerulosclerosis) [4–7]. In embryogenesis, *WT1* is necessary for sex determination – it controls expression of *SRY* (on chromosome Y) that together with *SOX9* initiates male gonadal differentiation. Postnatally, *WT1* is necessary for normal spermatogenesis and is highly expressed in both interstitial and germ cells [8]. This gene has been found to be inactivated in familiar Wilms tumors and its abnormal expression or mutations are associated with gonadal dysgenesis syndromes such as WAGR, Denys–Drash, and Frasier syndromes [9–11]. It has been extensively studied because of its supposed role in oncogenesis, as a potential prognostic factor, minimal residual disease marker and target of vaccination immunotherapy in various malignancies, particularly in acute leukemia [12,13]. *WT1* role in TGCT pathogenesis thus may be expected, however, only little is known about *WT1* in this context [14].

A number of genes have been studied in relation to TGCT predisposition, progression and treatment response/cisplatin resistance using classical molecular-biology approaches, genome-wide association studies, and recently massively parallel sequencing. Several genes of special interest have been identified. *TP53* tumor suppressor gene represents a crucial factor in DNA damage response and oncogenesis. While *TP53* mutations are the most common alterations in solid tumors, usually associated with aggressive characteristics and poor outcome, in TGCT they are rather rare events (up to 1–5%) [15] and *TP53* signaling pathway in TGCT may be functionally disrupted by different mechanisms, like interactions with other factors (MDM2, miRNA 371–373). As a result, an accumulation of inactive wild type p53 protein in the TGCT cytoplasm is often found [15–23]. *KRAS*, the most often mutated oncogene in solid tumors, is supposed to be involved in TGCT pathogenesis due to its deregulation resulting from the 12p amplification, a hallmark of TGCT [24,25]. *BRAF* mutations have long been presumed to be a prognostic factor in TGCT, as they were identified in significantly higher frequency in platinum-resistant tumors, being associated with microsatellite instability (MSI) and mismatch repair deficiency due to the lack of *hMLH1* expression [26]. Unfortunately, recent studies have not reproduced this observation [27]. *KIT* signaling pathway alteration is probably the key event in the early stages of TGCT development [28–30]. *KIT* mutations, amplifications or overexpression have been the most frequently detected alterations in TGCT, particularly in seminomas. They could be found already in pre-invasive lesions (intratubular germ cell neoplasia – ITGCNU), and represent also a potential druggable target [31,32].

The main objective of this study was to explore the role and impact of a novel factor – *WT1*, along with genes previously linked to TGCT including *TP53*, *RAS*, *BRAF*, *KIT*, in the pathogenesis and clinical outcome of TGCT, employing classic detection techniques – real-time quantitative PCR, immunohistochemistry, together with modern methods – massively parallel sequencing, on a prospectively enrolled cohort of patients as well as retrospectively gathered samples, to evaluate the feasibility, benefits and limits of these approaches in the search for novel prognostic factors and therapy targets in solid tumors, here in TGCT.

Material and methods

Samples

Cell lines

Three TGCT cell lines were cultured in a standard way, according to the supplier's recommendations. They were used for method optimization and as controls (details in Supplementary Material).

Patient samples

All samples were collected after ethical committee approval and informed consent of patients or their relatives.

During the years 2011–2015, 105 patients with a primary testicular tumor were enrolled on a prospective consecutive basis, including 52 seminomas, 49 non-seminomas, and 4 stromal tumors (Table S1). Fresh-frozen (FF) samples were collected from these tumors. In larger and heterogeneous tumors, 2 different tumor samples were taken from different tumor regions. If residual normal testicular tissue surrounding the tumor was preserved, it was also sampled as a control. The absence of invasive tumor cells in these controls was confirmed by histopathological examination of the samples; however, these samples often contained *in situ* lesions (intratubular germ cell neoplasia, ITGCNU). Further control samples were obtained from 77 patients in whom testicular surgery was performed for other diagnosis – prostate cancer (as a mean of hormonal treatment), inflammation, trauma, sex reassignment surgery, metastasis of other primary tumors (non-Hodgkin lymphoma, squamous cancer). In case of bilateral surgery, samples were taken from both testicles. Formalin-fixed paraffin-embedded (FFPE) tumor samples matched to FF samples were also available (e.g. for immunohistochemistry).

Additional search for TGCT biopsies deposited in hospital bio-banks was performed and FFPE samples from further 143 TGCT patients diagnosed in the years 2002–2012 with available informed consent could be used for the study. In some patients, several samples were examined (bilateral TGCT, primary tumor and its metastasis). Surrounding tumor-free testicular tissue or spermatic cord/epididymis was sampled as a control.

Peripheral blood of TGCT patients was collected and stored as another control.

Methods

RNA and DNA extraction

RNA and DNA from FF samples were extracted using RNeasy Mini Kit and QIAamp DNA Mini Kit (both Qiagen, Hilden, Germany). RNA and DNA from FFPE samples were extracted with High Pure RNA Paraffin Kit (Roche, Basel, Switzerland) and RecoverAll Total Nucleic Acid Isolation Kit for FFPE (ThermoFisher Scientific, Waltham, MA, USA). Their concentration, quality and integrity were evaluated by spectrophotometry – Nanodrop 2000 and Qubit 2.0 Fluorometer (both ThermoFisher Scientific), capillary electrophoresis – Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and by PCR [33]. The cDNA synthesis was performed using the iScript kit (Bio-Rad, Hercules, CA, USA) starting from 1 µg of total RNA. All procedures were carried out according to the manufacturers' instructions.

qPCR for total *WT1* and *WT1* isoforms

Real-time qPCR for total *WT1* and its isoforms with their absolute quantification using plasmid calibrators were of our own design, performed as described previously [34,35]. *WT1* expression was normalized to *ABL* control gene. Four main *WT1* isoforms that differ in the presence or absence of exon 5, encoding 17 amino acids [EX5], and so called KTS fragment at the end of exon 9, encoding 3 amino acids [KTS], were detected separately – variants A [EX5–/KTS–] (NM_000378), B [EX5+/KTS–] (NM_024424), C [EX5–/KTS+] (NM_024425) and D [EX5+/KTS+] (NM_024426).

Detection of *WT1* mutations

WT1 mutations were detected in exons 7 and 9, where the mutation hot spots are located, by classic Sanger sequencing as published previously [36].

Detection of *BRAF* 1799T>A (V600E) mutation

BRAF 1799T>A (V600E) point mutation was detected and quantified by TaqMan Mutation Detection Assay (ThermoFisher Scientific), and verified by KRAS-BRAF StripAssay (ViennaLab, Vienna, Austria), according to the manufacturers' instructions.

Immunohistochemical staining of *WT1* and p53 protein

The presence of *WT1* and p53 proteins in tissue sections prepared from FFPE tumor samples was evaluated by immunohistochemistry (details in Supplementary material).

The expression of p53 protein in cells was assessed semiquantitatively using the following scoring system: score 0 – totally negative or very weak positivity or positive in <10% of cells; score 1 – positive in 10–29% of cells; score 2 – positive in 30–49% of cells; score 3 – positive in 50% of cells or more.

Massively parallel sequencing (next generation sequencing, NGS)

Twenty six FFPE TGCT samples representative of different histologic subtypes, clinical stages and enrolling hospitals were subjected to amplicon sequencing using Ion AmpliSeq Comprehensive Cancer Panel (ThermoFisher Scientific) with all-exon coverage of over 400 genes involved in cancer pathogenesis, and Ion Torrent platform (ThermoFisher Scientific) (details in Supplementary material). Paired peripheral blood samples were processed in the same way, as germ-line controls.

Sequencing data were analyzed using Torrent Suite Software (ThermoFisher Scientific), FastQC (Babraham Bioinformatics, Cambridge, UK) [37], Qualimap (Max Planck Institute For Infection Biology, Berlin, Germany) [38], Ion Reporter Software (ThermoFisher Scientific) and Ingenuity Software (Qiagen), and visualized in Integrative Genomics Viewer (IGV) (Broad Institute, Cambridge, MA, USA [39]. The short variants (single nucleotide variants – SNVs, short Indels) as well as the copy number

variations (CNVs) of selected genes – *WT1*, *TP53*, *MDM2*, *ATM*, *KRAS*, *NRAS*, *HRAS*, *BRAF* and *KIT* – that passed QC and filtering criteria were detected (details in Supplementary material).

Statistical analysis

Final results were analyzed using statistical software – Excel (Microsoft, Redmond, WA, USA), Prism (GraphPad, La Jolla, CA, USA), and R (Vienna, Austria) [40]. Group comparisons of *WT1* and *p53* expression data were carried out using non-parametric tests – Mann–Whitney or Kruskal–Wallis with Dunn's multiple comparison post-test, Chi-square and Fisher's exact test. Gene variants data from the NGS study were normalized to the number of amino acids per gene and logarithmically transformed. The variations in gene variant/mutation frequencies were analyzed by Friedman rank sum test and pairwise comparisons by Wilcoxon signed rank test. The linkage among the mutated genes was tested by Spearman correlation coefficient. The differential gene mutation pattern between seminoma and non-seminoma was evaluated in linear mixed-effects model.

Results

WT1 aberrations in TGCT

WT1 expression was evaluated in FF samples (110 testicular tumors, 99 controls) (Table S2). The expression of total *WT1* (Figs 1 and S1) in both types of non-malignant control samples (non-malignant testes, tumor surroundings) was quite uniform and comparable. *WT1* levels in TGCT were significantly lower than in the controls ($p < 0.0001$), *WT1* being deeper under-expressed in seminomas than in non-seminomas. *WT1* expression in metastatic malignant tumors found in testes was similar to TGCT, while *WT1* levels in gonadal stromal tumors were higher, reaching values of non-malignant controls. Within paired samples, there was a constant difference of approximately 1 log between TGCT tumors and their non-malignant surroundings; in contrast, no major difference could be observed in between different tumor regions or bilateral non-malignant testes. In subgroup analysis, seminomas expressed significantly lower *WT1* levels than non-seminomas ($p = 0.0006$); other clinical parameters (e.g. age or clinical stage at diagnosis) did not prove to have an impact on total *WT1* expression. TGCT patients who relapsed showed *WT1* expression close to the limits of *WT1* ranges detected in all TGCT samples, as well as the respective clinical-pathology subgroups that stayed in remission: the expression was quite high in patients who relapsed after 1st line BEP (bleomycin/etoposide/cisplatin) chemotherapy given for an advanced stage II–III disease, but very low in patients with an early stage I TGCT who relapsed during watch-and-wait approach. The two patients in this cohort who died of TGCT expressed higher total *WT1* levels than the majority of TGCT.

WT1 isoform expression (Table S2, Figs 2 and S2) in non-malignant samples showed a common pattern regardless the type of the control, with the over-expression of variant D followed by variant B (both containing exon 5) and low levels of variants C and A (both lacking exon 5). In TGCT, there was a marked increase in the expression of variants A and C at the expense of variant B and especially D. Similarly altered expression pattern was present also in gonadal stromal tumors and all testicular germ cell lines, and was independent of the total *WT1* expression. While there was a pronounced difference in the ratio of exon 5-positive versus -negative splice variants between control and malignant samples ($p < 0.0001$), the ratios of KTS-positive and KTS-negative variants were remarkably stable across all sample types and individuals. No relation between *WT1* isoform expression pattern and other laboratory and clinical data was observed.

WT1 expression was also detected on the protein level by IHC staining (Fig. S3). *WT1* protein was present in normal seminiferous tubules, mostly in Sertoli cells. Normal germinal cells contained very low and variable amount of *WT1* protein, while TGCT cells were uniformly negative. In gonadal stromal tumors, *WT1* protein was

detectable and its expression varied according to the tumor differentiation grade.

No *WT1* mutations were found by direct sequencing of exons 7 and 9 in 48 tumor samples. Two types of single nucleotide polymorphisms (SNPs), both of unknown clinical significance, were identified: rs16754 – synonymous, in 16 (33%) patients; and rs5030274 – intronic, in 1 patient (2%). The former SNP was also detected in 2 of 3 TGCT cell lines analyzed, NCCIT and T-CAM. There was no correlation between the SNPs presence and expression of total *WT1* or its isoforms, nor was there any association between SNPs and other tumor/patient characteristics.

BRAF 1799T>A (V600E) mutation in TGCT

This specific point mutation was detected only in 2 (1%) of 210 tested samples, one case was a seminoma, the other a non-seminoma, both patients without a relapse event. None of the 3 TGCT cell lines harbored this mutation.

Expression of *p53* protein in TGCT

p53 protein expression was evaluated in 202 primary and 18 metastatic (mostly retroperitoneal lymph node metastases) TGCT tumors (Figs 3 and S3). In general, majority of samples revealed low intensity of *p53* protein expression – 79 samples of score 0, 82 of score 1. Only about one quarter of cases stained stronger for *p53* – 40 samples of score 2 and 19 of score 3. There was a profound difference in *p53* protein presence/expression between seminomas and non-seminomas ($p < 0.0001$), with prevailing low *p53* expression in seminomas and high-score *p53* expression occurring mostly in non-seminomas. Interestingly, also metastatic TGCT tumors exhibited low *p53* protein expression and we could document a significant decrease in detected *p53* protein between primary TGCT tumors and their distant metastases. However, there was no significant difference in *p53* expression between patients who stayed in complete remission and those who relapsed or died of TGCT, neither there was an association with other clinical factors.

WT1, *TP53*, *RAS*, *BRAF*, and *KIT* aberrations in TGCT-NGS analysis

Amplicon sequencing was performed in 26 FFPE TGCT samples and corresponding peripheral blood controls. The sequence quality (FastQC) and coverage parameters (Figs 4A–C and S4) confirmed primary data as relevant and acceptable for further analysis. A set of genes presumed to be crucial to TGCT pathogenesis and prognosis were selected and analyzed in detail, including *TP53* and its regulators *ATM* and *MDM2*, *KIT*, *RAS* genes – *KRAS*, *NRAS* and *HRAS*, *BRAF* and *WT1*. With the primary filter settings (variant allele ratio ≥ 0.1), 241 somatic single nucleotide variants (SNVs)/mutations, newly occurring in tumor samples and not present in germ-line controls, were identified. Among them, G→A and C→T transitions were highly prevailing. Due to concerns about possible artifacts involved, further filtering (variant allele ratio ≥ 0.3 for G→A and C→T transitions) was employed to increase the specificity of the findings. With these strict criteria, 47 SNVs were confirmed (Tables 1 and S3, Fig. 5). The medium variant allele frequency was 37%, with the range 10–100%. These were all base substitutions with mis-sense or nonsense functional effect. Some of these variants have been described before and are listed in dbSNP or COSMIC databases. No case of short insertions/deletions recognized by the analytic software could be confirmed by the visual inspection of the sequences – because the possibility that they may have arisen due to a technical error (e.g. indels within homopolymers or repeat regions, etc.) could be never definitely ruled out. In 10 out of 26 (38%) samples, no variants of the selected genes were detected; these were mostly samples with low quality DNA and therefore poorer coverage, where

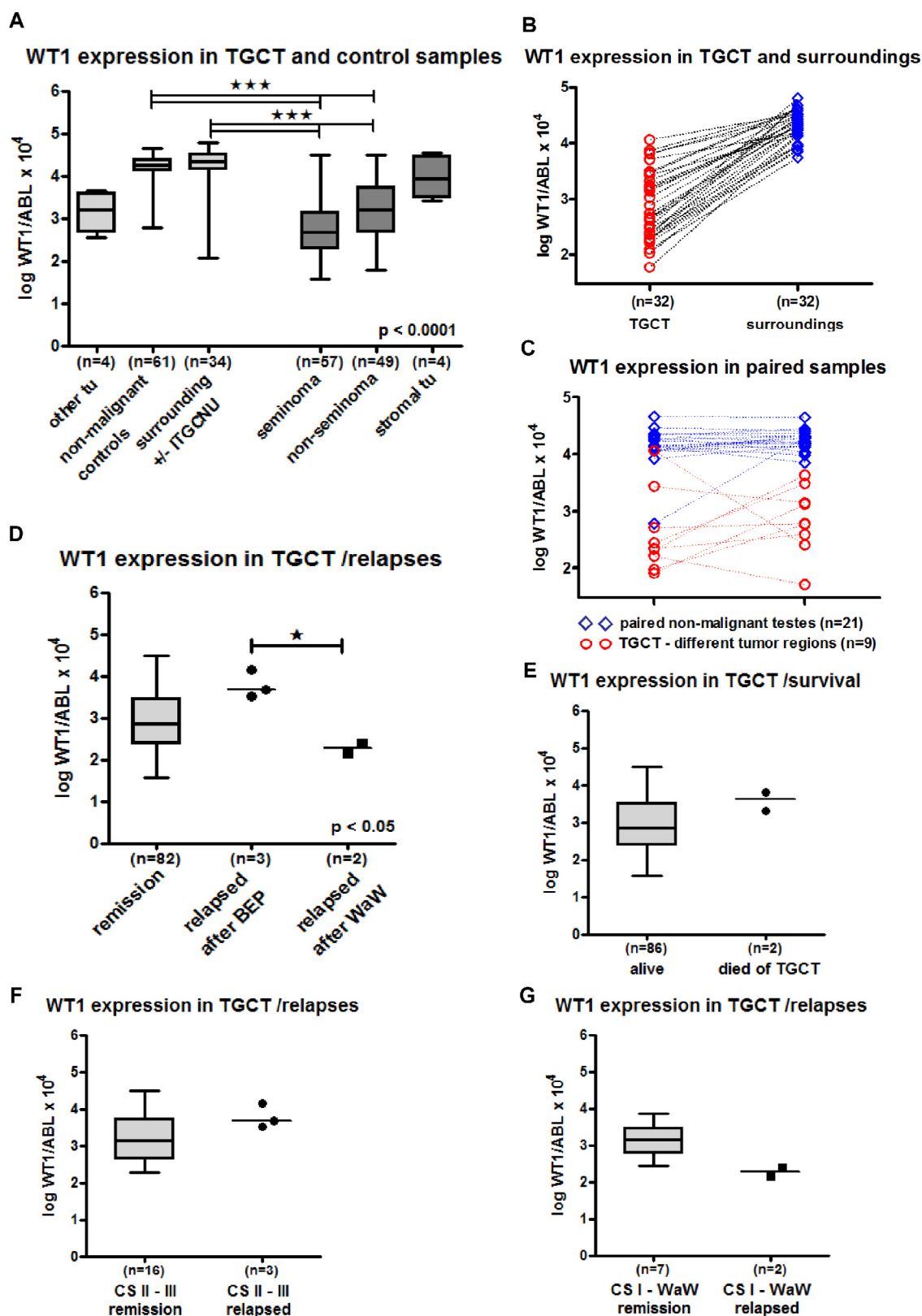


Fig. 1. Total WT1 expression in TGCT samples: (A) in TGCT and different controls; (B) in TGCT and the corresponding surrounding normal testicular tissue (may contain ITGCNU); (C) in paired samples, either bilateral non-malignant testes or different TGCT tumor regions; (D) in TGCT pts in remission vs. two different relapsed groups; (E) in TGCT pts surviving vs. those died of TGCT; (F) comparison of pts with TGCT of advanced stages II–III who after initial chemotherapy BEP stayed in remission vs. relapsed; (G) comparison of pts with early stage TGCT who on active surveillance stayed in remission vs. relapsed.

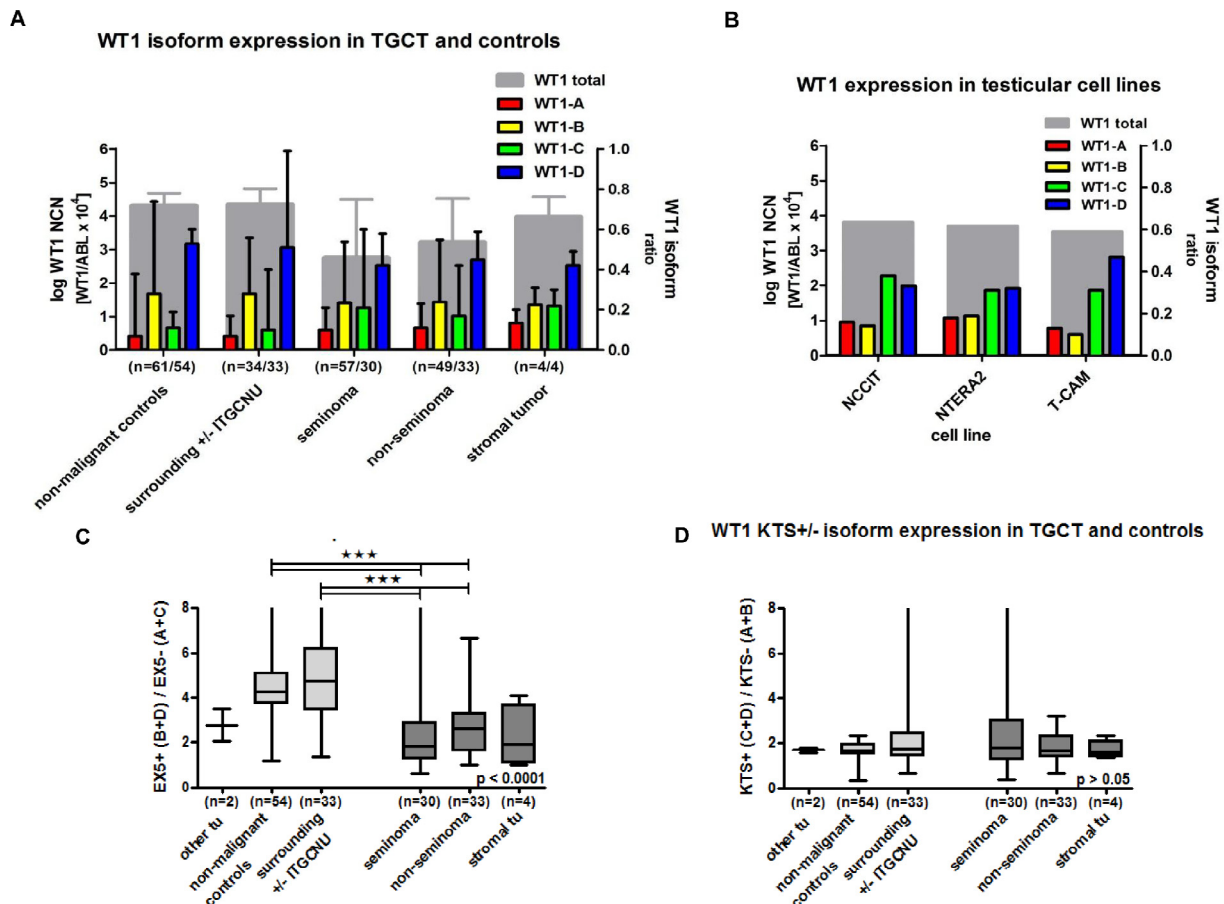


Fig. 2. Expression of WT1 isoforms (A) in TGCT and different controls (n = number of samples evaluated for total WT1/WT1 isoforms); (B) in TGCT cell lines; (C) WT1 exon 5+/- ratio in TGCT and controls; (D) WT1 KTS+/- ratio in TGCT and controls.

the variants did not pass the filtering criteria. In the remaining 16 samples, 1–10 (median 3) variants were found. Among them, one identical pathogenic variant of *KRAS* (p.Gly12Cys, COSM516) was identified in 2 different samples. The presence of this variant was confirmed also by Sanger sequencing. *ATM* variants were detected in the largest number of samples (at least one SNV found in 42% of samples), followed by *BRAF* (27%), *WT1* (23%) and *RAS* genes (19%). The highest number of variants per gene was also observed in *ATM*

gene – 21 (48% of all variants), which corresponds with its length. The variants were spread evenly over the whole gene/exons. No variants were detected in *TP53* and *KIT*. Even when the absolute number of variants was related to the gene exons/transcript length (expressed as the number of amino acids (AA) of the full-length gene transcript), the relative mutation frequency differ significantly among the genes (p = 0.0003). The frequency of variants was higher in *BRAF* (1.6 variants per 100 AA), followed by *RAS* (representing *KRAS*, *NRAS*

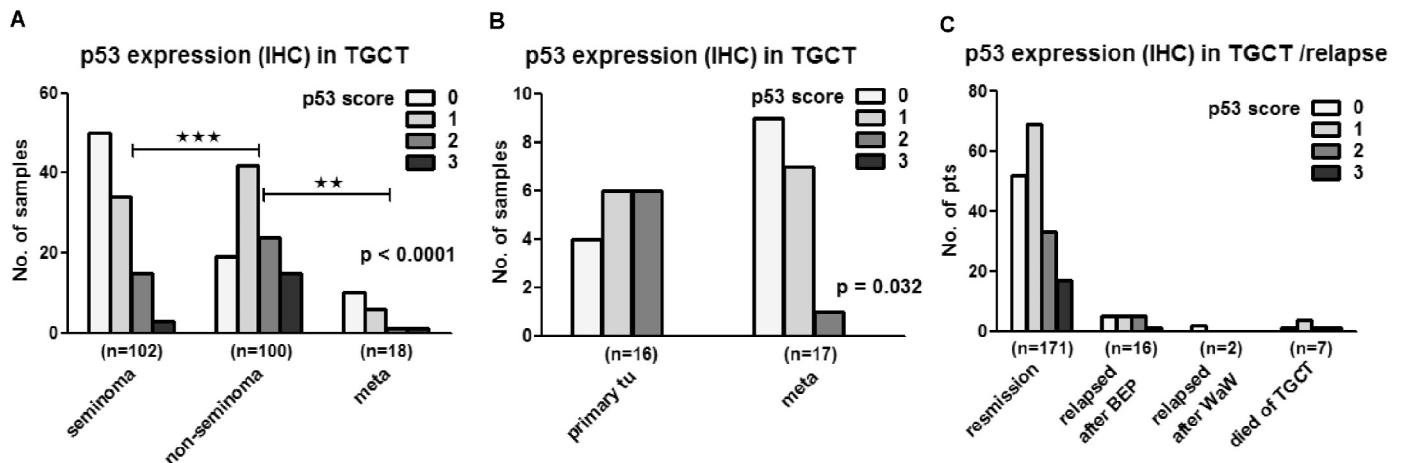


Fig. 3. p53 protein expression detected by IHC (A) in different TGCT samples; (B) in primary TGCT tumors and their corresponding distant metastases; (C) in TGCT pts in remission vs. relapsed or died of TGCT.

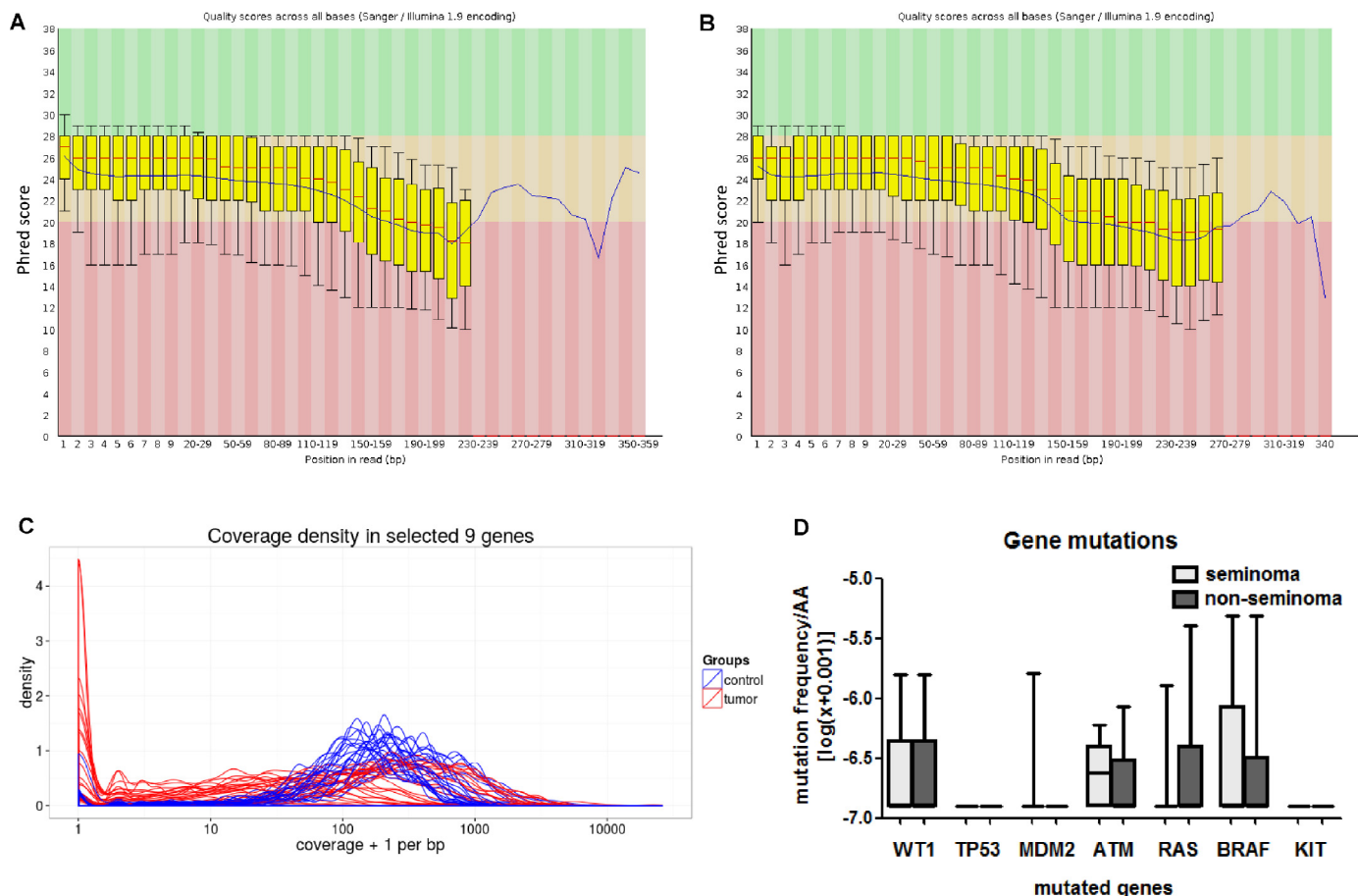


Fig. 4. Massively parallel sequencing performed as an amplicon sequencing with a comprehensive cancer panel (>400 genes) on Ion Torrent platform. Primary data analyzed by FastQC, per base sequence quality of (A) FFPE TGCT sample ID 1; and (B) its corresponding FF peripheral blood control; similar data for the other samples are in supplementary material, Fig. S4; (C) coverage density of the 9 selected genes – WT1, TP53, MDM2, ATM, KRAS, NRAS, HRAS, BRAF and KIT, according to the kernel density estimation, including bases with zero coverage; (d) mutation frequencies (gene mutations related to the number of amino acids of the full-length gene transcripts) detected in the selected genes.

and *HRAS* genes taken together) and *WT1* genes (1.2/100 AA both), and lower in *ATM* and *MDM2* genes (0.7 and 0.2/100 AA, respectively). The search for a potential linkage between mutated genes revealed a correlation of variant occurrence between the genes *BRAF*–*WT1* ($p = 0.001$).

There was no significant difference in variant frequency between seminoma and non-seminoma patients, although *BRAF* variants were more common in seminomas and *RAS* variants were prevailing in non-seminomas (Fig. 4D).

Copy number variations covering the selected genes were found in 2 samples (Table S3): 1 non-seminoma (ID 24) – with copy number loss in genes *KIT*, *WT1* and *ATM*; and 1 seminoma (ID 26) – with copy number gain in *KRAS*.

Discussion

Despite the increasing amount of knowledge about TGCT biology, there are several crucial points remaining to be addressed: what is the basis of their exceptional sensitivity to cisplatin and what causes the cisplatin-resistance associated with treatment failure; what prognostic factors can predict the risk of relapse and could be employed for patient stratification and individualization of the treatment; and, finally, what molecular aberrations may be used for an effective targeted therapy that could reverse the presently dismal outcomes of cisplatin-resistant patients. The novel high-throughput techniques like NGS bring a lot of promise of finding the answers

soon. On the other hand, the relatively low incidence of TGCT, low relapse rates, inconsistent treatment approaches across different centers and countries, and generally low availability of FF samples of these tumors in biobanks outside research studies – which are scarce, represent serious obstacles to the fast progress in this field. In this study, we have explored the feasibility, potential and limits of different approaches – FF/FFPE sample source, RNA/DNA-based, single-gene/cancer panel assays, and evaluated the role of novel factor – *WT1* gene, along with selected other genes, in TGCT pathogenesis and, possibly, in clinical management.

We have shown that *WT1* is under-expressed in TGCT in comparison to non-malignant testicular tissue. The IHC staining for *WT1* however revealed that the absence of *WT1*-highly expressing Sertoli cells in the tumor tissue may be partially responsible for this observation. Nonetheless, the difference in *WT1* expression between seminomas and non-seminomas and the profound alteration in *WT1* isoform expression pattern in testicular tumors that was present in both germ cell and stromal tumors as well as TGCT cell lines and was independent of total *WT1* levels, and also the relatively high rate of *WT1* mutations detected in TGCT by NGS, all point to the major role of *WT1* in TGCT. As a transcription factor that is also involved in mRNA editing, *WT1* controls the expression of a number of genes involved in the cell cycle, proliferation, differentiation and apoptosis. Its functions depend upon the balance of its isoforms (KTS insert alters the spacing between the protein's third and fourth zinc finger, thus changing the DNA recognition and binding ability; while

Table 1

Somatic single nucleotide variants (SNVs) in selected genes detected in TGCT patients by NGS.

ID	Histology	CS	OS	Total no. of SNVs	WT1	TP53	MDM2	ATM	RAS	BRAF	KIT
1	S	I	A	1				1			
3	S	I	A	3				1	1	1	
4	S	I	A	3				2, 1*			
6	NS	I	A	2	1					1	
7	S	II	A	3	1					2	
9	NS	I	A	2					2		
10	NS	I	A	10	1			3, 1*	2	2, 1*	
11	S	I	A	6	1			2		3	
16	S	I	A	1				1*			
18	NS	I	A	1					1		
20	NS	I	A	1				1			
21	S	II	A	3			1*	2			
22	NS	III	R, D	3	1			2			
23	S	I	A	4	1			2		1	
24	NS	I	A	3				2		1	
26	S	I	A	1					1		
Total				47	6	0	1	21	7	12	0
variants/100 AA					1.2	0.0	0.2	0.7	1.2	1.6	0.0
variants/1000 b					2.3	0.0	0.4	1.5	2.5	3.2	0.0
% of all variants					14	0	2	48	16	27	0
% of pts				62	23	0	4	42	19	27	0

ID – sample ID number, S – seminoma, NS – non-seminoma, CS – clinical stage, OS – overall survival, A – alive in remission, R – relapsed, D – died; variants/100 AA – number of variants per 100 amino acids (estimated from the full-length gene transcripts: *WT1* – 497 AA, *TP53* – 393 AA, *MDM2* – 491 AA, *ATM* – 3056 AA, *RAS* – 566 AA (*KRAS* 188 AA, *NRAS* 189 AA, *HRAS* 189 AA), *BRAF* – 766 AA, *KIT* – 976 AA), variants/1000 b – number of variants per 1000 bases of the sequenced area of the gene (*WT1* – 2588 b, *TP53* – 2048 b, *MDM2* – 2338 b, *ATM* – 13841 b, *RAS* – 2784 b (*KRAS* 909 b, *NRAS* 882 b, *HRAS* 993 b), *BRAF* – 3792 b, *KIT* – 4473 b); numbers in gene columns mean the number of variants where simple numbers mean missense alterations and numbers with * mean nonsense alterations. The summary and the statistics of the identified SNVs are presented in italics.

exon 5 functions as a transcription activation domain) and interactions with different co-factors (namely TET2) [35,41,42]. Imbalance in *WT1* expression and isoform ratio, may thus lead to a dysregulation of principal signaling pathways and contribute to the malignant transformation and growth.

We could see a non-significant trend toward increased *WT1* levels in higher TGCT stages, which was the main observation of the only previous study [14]. The prognostic value of *WT1* could not be confirmed so far due to the low relapse rates.

We have demonstrated that the specific *BRAF* V600E point mutation is infrequent in unselected TGCT patients regardless their relapse or survival status, which is in agreement with another recent study [27]. On the other hand, other *BRAF* mutations, as detected by NGS, seem to be the most common mutations in TGCT, particularly in seminoma patients.

RAS mutations (6 *KRAS* and 1 *NRAS* SNVs detected) as well as *KRAS* amplification were indeed frequent aberrations in our TGCT patients. Also in other TGCT studies, aberrations of these genes were repeatedly identified [27,43–45]. We found an identical mutation in the codon 12 in two patients; this activating mutation has been described as pathogenic in a number of solid and hematological malignancies and often associated with worse outcome/survival characteristics [24,25].

TP53 mutations are rare in TGCT and, in consistency with previous observations, were not reliably detected in our cohort of patients. Other NGS studies found them only in patients with cisplatin resistance or relapse after chemotherapy [44]. An interesting finding of our study is a significant decline in p53 protein expression in the cells of distant metastases in comparison to the primary TGCT, which may suggest its role in the metastatic spread or growth. As almost all these metastases were vital residues persisting after one or several lines of platinum-based chemotherapy, it more importantly indicates the association of p53 expression changes and cisplatin resistance. This well correlates with the NGS data and supports the role of altered p53 signaling in the exceptional platinum sensitivity in TGCT.

In recent studies, *KIT* mutations were detected in 10–20% of seminomas [43,45,46] and were recognized as one of the most common

mutations in TGCT. In our study, however, significant *KIT* variants were not found – even after manual check of the sequenced gene was performed in all samples to exclude possible technical errors or low quality results, which could influence the analysis. Although the stringent filtering criteria may lead to omitting SNVs that were present in lower allele frequencies, it still means that *KIT* mutations in our cohort of TGCT patients were quite rare in comparison with other genes, namely *KRAS* and *BRAF*.

NGS studies are carried out largely on FF samples; in TGCT, several such studies have been published so far [43,45–48]. However, the availability of FF TGCT samples is limited, as is the length of follow-up and the proportion of clinically interesting subgroups (relapsed, cisplatin resistant, bilateral and familial tumors, primary tumors and their metastases, etc.). The feasibility of FFPE samples for NGS studies may be therefore of interest. It is known that in FFPE tissues, DNA integrity depends on a number of factors, in particular the type of the fixative and the fixation time, the length and conditions of the storage, and the DNA extraction procedures [33]. Neutral-buffered formalin fixation and the storage up to 1–2 years may usually preserve the DNA in the quality sufficient for following molecular applications, including NGS. In our hands, the attempts of whole exome sequencing of few FFPE samples were not very successful, respectively yielded low cost-effective results. We could demonstrate a high degree of DNA disintegration in these often up to 10-year-old samples, with DNA fragments only around 100 bp long. Employing amplicon sequencing instead, we could obtain sequences of a good quality and coverage enabling further gene analyses in the vast majority of unselected FFPE samples. Apart from DNA integrity, chemical modifications in DNA, such as cross-linking, deamination and adducts, may occur during the FFPE process and affect the sequencing results. Contrary to DNA fragmentation, these may be quite difficult to identify, as they usually cannot be recognized by analysis software and on visual sequence inspection they may not be distinguishable from true mutations. As they mostly occur in lower allele frequencies – up to 20%, it is also hard to exclude them by Sanger sequencing because even real mutations occurring in similar frequencies are often below the detection threshold of this method. An abnormal incidence/ratio of certain

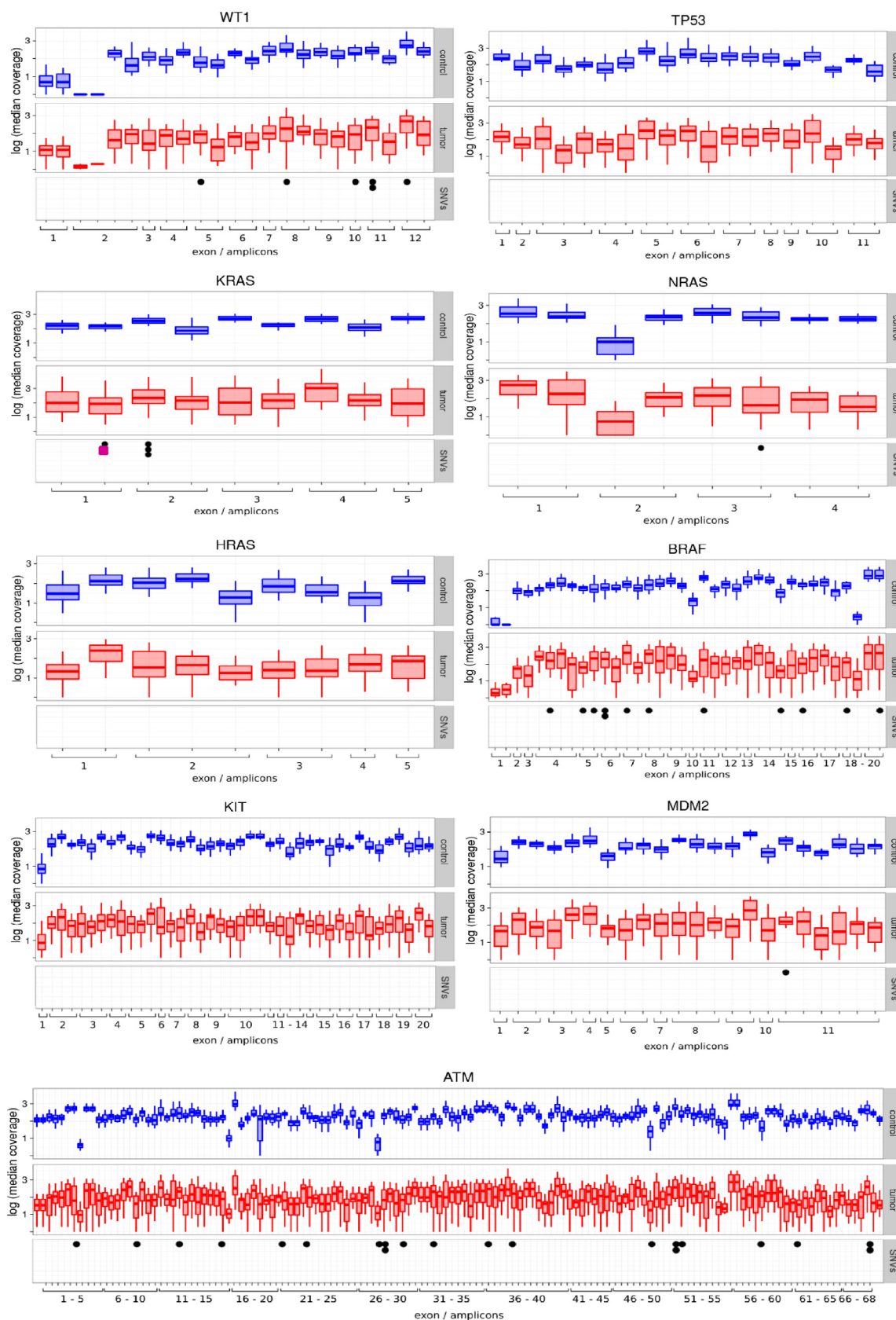


Fig. 5. Mutations (SNVs) detected in the selected genes in relation to the coverage of the corresponding regions. Boxplots show the distribution of the median sample coverage for individual amplicons; tumor samples in red, control samples in blue. Black dots represent the mutations detected in each amplicon of tumor samples, purple square means identical mutations present in 2 different samples. Ticks on the x-axis mean the sequenced amplicons that are grouped into the exons indicated by numbers. No correlation between the mutation density in the different gene regions/amplicons and the coverage density has been identified. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

types of substitutions may suggest the presence of artifacts of this type. Within our results, G→A and C→T transitions were present much more often (more than 10×) than other substitutions. They may occur due to guanine or cytosine deamination and cause false positive results, as have been already described [49]. Increasing the filtration threshold for variant allele frequency from 10 to 30% in case of these substitutions should solve this problem, although it is at the expense of probable loss of some real mutations. However, in this type of study, an improved specificity and reliability of the data is superior to the sensitivity of the detection. The sensitivity of amplicon sequencing of FFPE samples is obviously lower than standard WES of FF samples, with certain limits that have to be respected. Despite that, it may add valid and valuable information to our expanding knowledge of TGCT biology and facilitate the translation into the clinical practice.

In summary, we have identified *WT1* gene as a novel factor involved in TGCT pathogenesis, where it may act, similarly to Wilms tumors, as a tumor suppressor gene. The study has shown the different biologic nature of the two types of relapses occurring in TGCT patients, which is not a common situation in other malignancies and is important to respect in future studies searching for new prognostic factors in TGCT; with *WT1* itself having a prognostic potential. A role of *TP53* in TGCT metastatic spread and cisplatin resistance has been implied. Finally, we have demonstrated the feasibility of massively parallel sequencing of FFPE TGCT samples, which may extend the opportunities of exploring the TGCT biologic landscape and discovering molecular aberrations with clinical impact.

Funding

This project was supported by grants from the Internal Grant Agency of the Czech Ministry of Health (IGA NT/12414-5), Charles University Grant Agency (GAUK 56413), Czech Ministry of Education, Youth and Sports (NPU I LO1604) and University Hospital Motol, Prague (00064203FNM).

Acknowledgments

We thank to Ondrej Hes, Sikl's Institute of Pathology, University Hospital Lochotin, Pilsen, Martin Syrucek, Department of Pathology, Na Homolce Hospital, Prague, Petr Hrabal, Department of Pathology, Central Military Hospital, Prague, Kamila Benkova, Department of Pathology, Na Bulovce Hospital, Prague, and Petra Berouskova, Department of Pathology, Regional Hospital Kladno, all from the Czech Republic, for their contribution with TGCT samples to this study; and Leendert Looijenga, Department of Pathology, Erasmus MC-University Medical Center Rotterdam, The Netherlands, for his kind donation of T-CAM cell line. The NGS sequencing was performed on Ion Proton Sequencer in Gennet, Prague, with the kind permission and assistance of Martina Putzova. We also thank to Martina Slamova, CLIP, and Vaclav Capek, Centre of Bioinformatics, both 2nd Faculty of Medicine, Charles University, Prague, for help with sample processing and statistical analysis of the data, respectively.

Conflicts of interest

All authors declare no conflicts of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.04.016.

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Polyclonal, newly derived T cells with low expression of inhibitory molecule PD-1 in tonsils define the phenotype of lymphocytes in children with Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome

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ARTICLE INFO

Article history:

Received 13 August 2014

Received in revised form

15 December 2014

Accepted 8 January 2015

Available online 3 February 2015

Keywords:

PFAPA

Autoinflammatory disease

Flow cytometry

T lymphocyte

PD-1

TREC

KREC

T-cell receptor gene rearrangement

Tonsillectomy

Chemokine

ABSTRACT

Purpose: PFAPA syndrome is a benign, recurrent inflammatory disease of childhood. Tonsillectomy is one of the therapeutic options with a yet unexplained biological mechanism. We tested whether specific lymphocyte subsets recruited from blood to human tonsils participate in PFAPA pathogenesis.

Methods: Paired tonsils/peripheral blood (PB) samples were investigated (a) from children with PFAPA that successfully resolved after tonsillectomy ($n = 10$) (b) from children with obstructive sleep apnoea syndrome as controls ($n = 10$). The lymphocyte profiles were analysed using 8-colour flow cytometry, immunoglobulin (IGH) and T-cell receptor (TCR) gene rearrangements via PCR and next generation sequencing; a TREC/KREC analysis was performed using qPCR.

Results: The PFAPA tonsils in the asymptomatic phase had a lower percentage of B-lymphocytes than controls; T-lymphocyte counts were significantly higher in PB. The percentages of cytotoxic CD8pos T-lymphocytes were approximately 2-fold higher in PFAPA tonsils; the transitional B cells and naïve stages of both the CD4pos and CD8pos T-lymphocytes with a low expression of PD-1 molecule and high numbers of TREC were also increased. With the exception of elevated plasmablasts, no other differences were significant in PB. The expression levels of CXCL10, CXCL9 and CCL19 genes were significantly higher in PFAPA tonsils. The IGH/TCR pattern showed no clonal/oligoclonal expansion. DNA from the Epstein-Barr virus, Human Herpesvirus-6 or adenovirus was detected in 7 of 10 PFAPA tonsils but also in 7 of 9 controls. **Conclusions:** Our findings suggest that the uninhibited, polyclonal response of newly derived lymphocytes participate in the pathogenesis of PFAPA. Because most of the observed changes were restricted to tonsils and were not present in PB, they partly explain the therapeutic success of tonsillectomy in PFAPA syndrome.

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1. Introduction

PFAPA syndrome in children ranks among the most common periodic autoinflammatory diseases. In 1987, PFAPA was

first described by Marshall according to its most characteristic symptoms: periodic fever, pharyngitis, aphthous stomatitis and cervical lymphadenitis. The diagnosis is made partly by exclusion; the diagnostic criteria for PFAPA syndrome modified by Thomas et al. (Thomas et al., 1999) include the following: (1) onset of disease prior to 5 years of age, (2) the absence of upper respiratory tract infection with at least one of the following symptoms: aphthous stomatitis, cervical lymphadenitis, pharyngitis, (3) asymptomatic interval between episodes, (4) exclusion of cyclic neutropenia and

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(5) normal growth and development. Additional features such as vomiting, nausea, fatigue, abdominal pain and arthralgia may occur (Hofer et al., 2014). The aetiology and pathogenesis of the syndrome remains unknown; a dysregulation of the immune system or abnormal response to an unknown infection trigger is suspected. Laboratory findings in peripheral blood (PB) during fever flares revealed elevated white blood cell counts (WBC) that was caused mostly by the increased neutrophil and monocyte counts accompanied by decrease in lymphocytes and eosinophils. Elevation of erythrocyte sedimentation rate, of C-reactive protein and of serum amyloid A was recorded during PFAPA flares (Thomas et al., 1999; Brown et al., 2010; Feder and Salazar, 2010; Kolly et al., 2013; Stojanov et al., 2006; Stojanov et al., 2011). Investigation of classic proinflammatory cytokines in sera revealed elevated serum concentration of IL-6, but unchanged levels of TNF- α and IL1 β approximately 15 h after the onset of the fever (Brown et al., 2010). The levels of lymphocyte activator IL-7 and Th17-associated cytokine IL17 were decreased both in febrile and in afebrile sera compared to controls in the same study (Brown et al., 2010). Another study exploring cytokine production came to slightly different results, showing increased plasma concentration of IL-1 β , IL-6, TNF- α and IL-12p70 after fever onset, but also in asymptomatic periods compared to controls (Stojanov et al., 2006). Increased percentages of CD4⁺ and CD8^{pos} T cells produced IFN- γ after stimulation compared to controls (Stojanov et al., 2006). Both studies concluded that the cytokine pattern is typical for an IFN- γ -dependent (Th1) inflammatory response. A study by Stojanov et al. (Stojanov et al., 2011) was able to distinguish PFAPA flares from asymptomatic period samples, control samples and also from hereditary periodic fever (HPF) flares based solely on the gene expression profile. During PFAPA flares, complement, IL-1-related and IFN-induced genes were significantly overexpressed. The gene expression profile during asymptomatic periods was indistinguishable from that of healthy children. PFAPA flares were accompanied by increased protein levels of chemokines known as the T cell chemoattractants IP-10/CXCL10, MIG/CXCL9 and MIP-1 β /CCL4. The most recent study suggested that dysregulated IL-1 β monocyte production is linked to PFAPA syndrome (Kolly et al., 2013).

The optimal treatment method for PFAPA syndrome remains a matter of debate. The administration of antibiotics and non-steroidal anti-inflammatory agents has been considered ineffective (Thomas et al., 1999). A single dose of prednisone aborts PFAPA flares but does not prevent their recurrence (Feder and Salazar, 2010; Berlucchi et al., 2003; Wurster et al., 2011). The administration of the recombinant IL-1R antagonist anakinra was successful in all five patients (based on the findings of above mentioned study) (Stojanov et al., 2011). A positive effect of tonsillectomy has been documented in several studies (Feder and Salazar, 2010; Stojanov et al., 2011; Garavello et al., 2009; Krol et al., 2013; Peridis et al., 2010a), although the pathogenetic background for this therapeutic intervention is unknown. Because tonsils are primarily a lymphoid tissue and because the gene expression profiles revealed the involvement of adaptive immunity, we decided to evaluate the perturbation of lymphocyte composition and function within tonsils removed as a treatment for PFAPA treatment. As anticipated in a previous study describing elevated levels of T cell chemoattractants in peripheral blood (Stojanov et al., 2011), we show that T cells, especially early developmental stages of CD8^{pos} cytotoxic and CD4^{pos} helper T cells with low expressions of inhibitory molecule PD-1, are more abundant in the tonsils of patients with PFAPA. The activation of the immune system by a virus could be a tempting explanation of tonsillar pathology; we found the DNA of common viruses in most PFAPA tonsils. However, the same viruses were also present in control tonsils. Interestingly, most of the observed differences in

our study were limited to tonsils and were not present in the blood.

2. Patients and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of 2nd Faculty of Medicine and Motol University Hospital in Prague, Czech Republic. The parents of the patients were informed about the aim of the study and gave their written consent. All clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

2.2. PFAPA and control samples

Ten children with PFAPA syndrome (5 boys, 5 girls) and 10 children with obstructive sleep apnoea syndrome (control group) underwent elective tonsillectomy with or without adenoidectomy at the ENT Department of the 2nd Faculty of Medicine, Charles University in Prague, between October 2010 and May 2013. The identification of PFAPA patients, diagnosis and follow up of the children with PFAPA syndrome were performed in cooperation with the Periodic Fever Clinic of the Pediatric Rheumatology Unit, General University Hospital in Prague. The children with tonsillectomy due to PFAPA syndrome consisted of three groups: (1) the patients whose parents opted for tonsillectomy as a first line treatment (3 patients); (2) the patients treated with episodic prednisone whose disease duration and episode frequency were an unacceptable burden for the family (4 patients) and (3) the patients treated with prednisone with increasingly frequent episodes (3 patients). Tonsillectomy was performed during symptom-free intervals. The cold knife method for tonsillectomy was employed, and adenoidectomy was performed if needed. The patients were followed for a minimum of 6 months after tonsillectomy: initially during common postoperative ENT follow up then via telephone contact. A sample of the peripheral blood was obtained during the tonsillectomy procedure.

2.3. Diagnostic algorithm

In order to exclude other causes of recurrent fevers all patients underwent general pediatric and ENT assessments.

The case history inquired was:

- age at the first manifestation of the symptoms
- age at diagnosis
- duration of fever episodes
- interval between fever episodes
- clinical findings during fever episodes: pharyngitis, cervical lymphadenopathy, aphthous stomatitis, arthralgia, abdominal pain and vomiting.
- clinical status during afebrile intervals

Laboratory evaluation:

- erythrocyte sedimentation rate, full blood count with differential, liver and kidney function tests, anti streptolysin O.
- nose and throat culture
- serum immunoglobulin (Ig) levels (namely IgG, IgA, IgD), herpetic virus serology and mevalonic acid in urine collected during the febrile episode (Krol et al., 2013).

Finally, clinical diagnosis of PFAPA syndrome was confirmed by a pediatric rheumatologist in the Periodic Fever Clinic.

2.4. Flow cytometry

Peripheral blood was collected into EDTA. Mononuclear cells were isolated via Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation or isolated via gentle shaking from manually dissociated tonsillar tissue. Dry pellets of the mononuclear cells were stored at -80°C for subsequent DNA analysis. Flow cytometry (FC) analysis was performed on a modified BD LSR II (BD Bioscience, San Jose, CA, USA) instrument equipped with a standard blue laser (488 nm, 20 mW), a red laser (633 nm, 25 mW) and an upgraded diode violet laser (405 nm, 120 mW, Omicron Laserage Laserprodukte GmbH, Rodgau, Germany). The lymphocyte subsets were evaluated using the following antibody combinations: T-cell panel: CD27 Pacific Blue, CD8 Horizon-V500, CD28 Alexa488, CD38 PE, CD3 ECD, CD45RA PE-Cy7, CD279 APC and CD4 Alexa700. B-cell panel: (CD27 Pacific Blue, IgM FITC, CD24 PE, IgD Qdot605, CD20 PerCP, CD19 PE-Cy7, CD21 APC and CD38 Alexa700) as described previously (Vlkova et al., 2010). The data were analysed using FlowJo software (TreeStar, Ashland, OR, USA). The left and right tonsils were analysed separately, and average values were used for further statistical evaluation because we observed no lateral skewing of the lymphocyte proportions.

2.5. Immunoglobulin and T-cell receptor gene rearrangements

DNA from the whole blood and from tonsils was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The clonality of the immunoglobulin heavy chain (IGH), TCR beta (TCRB) and TCR gamma (TCRG) rearrangements were assessed using multiplex PCR as described previously (Pongers-Willemse et al., 1999; van Dongen et al., 2003). The PCR products were analysed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). TCRG rearrangement profiling was performed on the DNA level via Immunoseq (Adaptive Biotechnologies, Seattle, USA) using next generation sequencing and the cloud-based analysis of multiplex PCR products as described previously (Sherwood et al., 2011).

2.6. TREC and KREC detection

The albumin gene level was quantitatively detected in isolated samples using qPCR (Pongers-Willemse et al., 1998) and standard dilution series derived from Human Genomic DNA with a known starting concentration of 200 ng/ μl (Roche, Basel, Switzerland). The T-cell receptor excision circles (TREC) and kappa-deleting element recombination circles (KREC) were used to detect proliferation history of T and B lymphocytes, respectively. TREC and KREC levels were assessed separately using cloned plasmid standards as previously described (van Zelm et al., 2007; Weinberg et al., 2001). The results were expressed as the number of TREC (KREC) copies per 1 microgram of DNA (Fronkova et al., 2014).

2.7. Detection of viral DNA

The viral loads of EBV, CMV, HHV-6 and adenoviruses were detected after DNA extraction using previously published qPCR assays based on a dilution series of cloned plasmid standards (Claas et al., 2005; Gautheret-Dejean et al., 2002; Kimura et al., 1999; Tanaka et al., 2000), and viral loads were normalised to quantified albumin genes (Pongers-Willemse et al., 1998) as described previously (Hubacek et al., 2009).

2.8. Gene expression analysis

Total RNA from frozen tonsils (8x PFAPA, 7x controls) was isolated using MELTTM Total Nucleic Acid Isolation System (Life

Technologies, Waltham, MA, USA). Real-time PCR was performed in duplicates using a dilution series of mixed PB cDNA's from five healthy donors. TaqMan[®] gene expression assays for CXCL1, CXCL9, CXCL10, CXCL12, CXCL13 and CCL19 with an ABI PRISM[®] 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) were used and the results were related to the level of *abl* control gene expression.

2.9. Statistical analysis

All statistical analyses and graphs were generated using the R statistical environment. The distribution of variables between groups was assessed using Fisher's exact test. The Wilcoxon-Mann-Whitney test was used to estimate the significance of the differences concerning continuous values. A classification tree based on information from all measured T-lymphocyte populations was created; the classification attributes and their thresholds were chosen using a C4.5 algorithm implemented in the R package RWeka (Ian et al., 2005; Hornik et al., 2009). This algorithm chooses the decision node so that the resulting splitting maximises the normalised information gain.

3. Results

3.1. Clinical course pre-and post-tonsillectomy

The median age at the first manifestation of the PFAPA symptoms was 27 months (range 18–60), and the median age at diagnosis was 39 months (range 14–78). The median duration of fever flare was 4.5 days (range 2–7) with a median interval between attacks of 4 weeks (range 2–7). Tonsillectomy was performed at a median age of 66 months (range 36–96). Pharyngitis was observed in all 10 patients, aphthous stomatitis in 4 patients (40%) and cervical adenitis in 5 patients (50%) during PFAPA flares. Arthralgia was observed in 3 patients (30%), abdominal pain in 1 patient (10%) and vomiting in none of the patients. 100% of the PFAPA patients had complete remission of the symptoms for at least 8 months after surgery. The median length of post-tonsillectomy follow up was 18.5 months (range 8–29). None of the patients had postoperative complications. The PFAPA tonsils were macroscopically similar to controls and the histology results of both PFAPA tonsils and controls were reported as chronic tonsillitis or chronic lacunar tonsillitis. The PFAPA tonsil cultivations were negative for bacteria in all patients.

3.2. T lymphocyte subsets

All measured values were highly concordant for tonsil pairs; therefore, we used the average values for further analyses. Compared with the controls, there was a slightly higher percentage of CD3^{pos} T lymphocytes in the peripheral blood of PFAPA patients (median 66% in PFAPA vs. 59% in controls, $p=0.01$, Fig. 1A). This trend was also present in tonsils but did not reach statistical significance (median 36% in PFAPA vs. 31% in controls, $p=0.08$, Fig. 1A).

The tonsils of PFAPA patients differed from those of controls in the proportion of the main T lymphocyte subsets (Fig. 1B). The PFAPA tonsils contained more CD8^{pos} cytotoxic T lymphocytes (PFAPA: median 6% of all lymphocytes; controls: median 3%; $p=0.007$). The CD4/CD8 ratio was significantly lower in PFAPA (PFAPA: median 4.2; controls: median 6.1, $p=0.02$). The proportion of CD4/CD8 double negative CD3^{high} cells corresponding mainly to gamma delta T lymphocytes was slightly increased in the PFAPA tonsils (median 1.2% of the CD3^{pos} cells in PFAPA vs. median 0.57% in controls, $p=0.02$, Table 1).

Supplementary Fig. 1 shows the gating strategy for defining four developmental stages of CD4^{pos} and CD8^{pos} T lymphocytes based

Table 1

	TONSIL - CTRL			TONSIL - PFAPA			Wilcoxon test	BLOOD - CTRL			BLOOD - PFAPA			Wilcoxon test			
	Median	(10–90 percentile)		Median(10–90 percentile)			p-value	Median (10–90 percentile)			Median	(10–90 percentile)		p-value			
Abs Lymphocytes (x 10e6/ml)	NA	NA		NA	NA		NA	Abs Lymphocytes (x 10e6/ml)	2.70	(2.36–3.86)		n = 7	3.30	(2.66–4.08)		n = 7	n.s.
CD19+ of Lymph	65.9	(58.2–72.53)	n = 6	54.4	(47.53–61.34)	n = 7	0.022	CD19+ of Lymph	18.9	(11.02–31.55)	n = 8	12.2	(11.4–17.18)	n = 9	n.s.		
CD3+ of Lymph	30.9	(25.38–36.01)	n = 10	36.3	(27.57–44.5)	n = 10	n.s.	CD3+ of Lymph	59.4	(44.09–66.32)	n = 10	66.2	(61.01–67.16)	n = 10	0.019		
CD4+ of Lymph	25.1	(20.84–30.65)	n = 10	26.4	(20.53–32.03)	n = 10	n.s.	CD4+ of Lymph	27.9	(17.96–35.69)	n = 10	30.7	(23.72–40.18)	n = 10	n.s.		
CD8+ of Lymph	3.14	(2.31–5.53)	n = 10	5.78	(4.02–8.65)	n = 10	0.007	CD8+ of Lymph	19.8	(12.54–27.54)	n = 10	20.6	(17.03–30.71)	n = 10	n.s.		
CD4/CD8	6.1	(4.77–9.28)	n = 10	4.2	(2.38–6.26)	n = 10	0.019	CD4/CD8	1.4	(0.92–2.16)	n = 10	1.4	(0.88–2.14)	n = 10	n.s.		
CD3 + DN of T-cells	0.57	(0.37–1.22)	n = 8	1.22	(0.91–1.74)	n = 9	0.021	CD3 + DN of T-cells	7.75	(3.72–9.85)	n = 10	7.85	(6.15–13.5)	n = 10	n.s.		
CD4+ Naive of Lymph	3.6	(2.76–6.94)	n = 10	6.3	(4.31–9.39)	n = 10	0.015	CD4+ Naive of Lymph	19.2	(11.8–25.68)	n = 10	21.1	(15.75–29.26)	n = 10	n.s.		
CD4+ Central Memory of Lymph	21.9	(16.06–29.02)	n = 10	20.0	(17.65–22.09)	n = 10	n.s.	CD4+ Central Memory of Lymph	7.62	(4.64–10.3)	n = 10	8.34	(6.5–10.32)	n = 10	n.s.		
CD4+ Effector Memory of Lymph	1.1	(0.55–1.71)	n = 10	0.9	(0.55–2.52)	n = 10	n.s.	CD4+ Effector Memory of Lymph	1.0	(0.59–1.72)	n = 10	1.1	(0.34–2.34)	n = 10	n.s.		
CD4+ TEMRA of Lymph	0.08	(0.05–0.29)	n = 10	0.06	(0.04–0.19)	n = 10	n.s.	CD4+ TEMRA of Lymph	0.04	(0.02–0.08)	n = 10	0.07	(0.03–0.3)	n = 10	n.s.		
CD4 + PD1hi of Lymph	10.1	(7.53–11.59)	n = 10	5.9	(4.42–8.91)	n = 10	0.001	CD4 + PD1hi of Lymph	0.0	(0–0.07)	n = 10	0.0	(0–0.06)	n = 10	n.s.		
CD8+ Naive of Lymph	2.085	(1.43–3.1)	n = 10	3.84	(2.4–6.8)	n = 10	0.005	CD8+ Naive of Lymph	12.2	(7.97–17.38)	n = 10	13.1	(9.51–15.49)	n = 10	n.s.		
CD8+ Central Memory of Lymph	1.2	(0.73–2.36)	n = 10	1.5	(1.05–4.43)	n = 10	n.s.	CD8+ Central Memory of Lymph	3.8	(2.09–8.25)	n = 10	4.8	(3.39–8.17)	n = 10	n.s.		
CD8+ Effector Memory of Lymph	0.12	(0.03–0.21)	n = 10	0.08	(0.06–0.35)	n = 10	n.s.	CD8+ Effector Memory of Lymph	0.48	(0.12–1.02)	n = 10	0.78	(0.32–1.5)	n = 10	n.s.		
CD8+ TEMRA of Lymph	0.0	(0.02–0.07)	n = 10	0.0	(0.02–0.13)	n = 10	n.s.	CD8+ TEMRA of Lymph	1.2	(0.48–3.17)	n = 10	1.9	(0.26–7.53)	n = 10	n.s.		
Transitional of B-cells	0.12	(0.04–0.2)	n = 6	0.54	(0.07–1.04)	n = 7	0.035	Transitional of B-cells	5.19	(2.32–7.9)	n = 8	7.07	(2.92–13.96)	n = 9	n.s.		
Naive/Immature of B-cells	63.0	(59.2–65.83)	n = 6	69.7	(57.89–76.79)	n = 7	n.s.	Naive/Immature of B-cells	57.0	(52.57–59.62)	n = 8	61.1	(53.04–69.2)	n = 9	n.s.		
Ig switched of B-cells	53.5	(40.9–60.6)	n = 6	38.2	(31.22–45.02)	n = 7	0.035	Ig switched of B-cells	19.3	(11.81–36.44)	n = 7	16.2	(11.24–29.07)	n = 8	n.s.		
Plasmablasts of B-cells	2.0	(1.5–2.73)	n = 6	1.1	(1.02–1.53)	n = 7	0.012	Plasmablasts of B-cells	1.2	(0.49–2.08)	n = 8	3.0	(1.22–4.98)	n = 9	0.018		
IgM negative CD27 negative of B-cells	29	(20–38.525)	n = 6	18.8	(17.45–25.27)	n = 7	0.035										

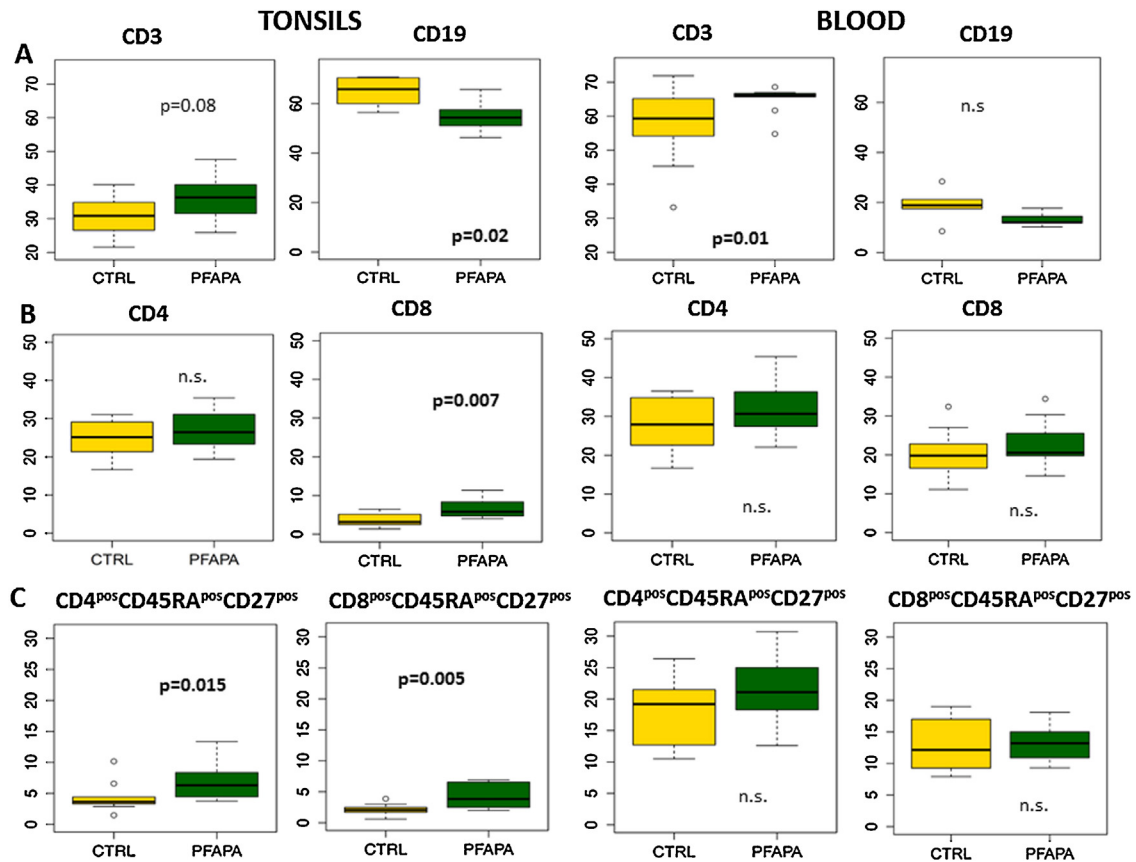


Fig. 1. Lymphocyte subsets in PFAPA vs. controls. Percentages from all lymphocytes are shown in all graphs. CD19^{pos} B lymphocytes and CD3^{pos} T lymphocytes (A), CD4^{pos} helper and CD8^{pos} cytotoxic T lymphocytes (B), naïve helper CD4^{pos} CD45RA^{pos}CD27^{pos} T cells and naïve cytotoxic CD8^{pos}CD45RA^{pos}CD27^{pos} T cells (C) are shown.

on the expression of CD45RA and CD27 markers in both tonsils and blood (Mahnke et al., 2013). The helper T cell lymphocyte pool was primarily composed of memory CD45RA^{neg} cells in both the PFAPA and controls, but the proportion of naïve CD45RA^{pos}CD27^{pos} cells was significantly higher in the PFAPA tonsils (PFAPA: median 6% of all lymphocytes; controls: median 4%, $p=0.015$, Fig. 1C). The percentage of naïve CD8^{pos} CD45RA^{pos}CD27^{pos} cells was significantly elevated in PFAPA tonsils (PFAPA: median 4% of all lymphocytes; controls: median 2%, $p=0.005$, Fig. 1C). The proportion of activated HLA-DR^{pos} cells in the PFAPA tonsils was comparable with that of controls for both the CD4^{pos} and CD8^{pos} T lymphocytes (data not shown).

In agreement with the study of Wang et al. (Wang et al., 2011), we found a heterogeneous expression of PD-1 molecule on CD4^{pos} T lymphocytes (Fig. 2A) in tonsils, being the highest (PD-1^{high}) in memory CD45RA^{neg} cells. The percentage of CD4^{pos} PD-1^{high} CD45RA^{neg} cells was significantly lower in the PFAPA tonsils than in the controls (median 6% of all lymphocytes in PFAPA vs. 10% in controls, $p=0.001$, Fig. 2B). Importantly, all observed differences in the T lymphoid subpopulations between PFAPA and controls were limited to tonsils. The blood samples taken during tonsillectomy did not show any significant differences regarding T lymphocyte subsets. Absolute lymphocyte counts in PB were similar to controls in PFAPA (Table 1). Analysis of total cell numbers in each T cell population confirmed higher numbers of PB T lymphocytes in PFAPA ($p=0.04$, Wilcoxon test, data not shown). Similarly to relative numbers, absolute counts of all analysed T lymphocyte subsets did not differ from controls (data not shown). In order to understand which parameters were different in PFAPA vs. controls an unsupervised classification tree based on information from all measured T-lymphocyte populations both in blood and tonsils was created.

The algorithm chose the CD4^{pos}PD-1^{high} percentage of all lymphocytes in tonsils combined with the percentage of CD8^{pos} cells of all lymphocytes in tonsils as the best predictor of PFAPA phenotype. The following classification was suggested: CD4^{pos}PD-1^{high} T cells from tonsillar lymphocytes < 9.25% and CD8^{pos} T cells from tonsillar lymphocytes > 3.46% (Fig. 3). This classification correctly assigned all but one patient to the respective categories (PFAPA vs. controls) in our limited cohort.

We asked whether the elevated numbers of respective T cell subsets were caused by their proliferation. The numbers of TREC per microgram of DNA did not differ between PFAPA and controls (Supplementary Fig. 3), which indicates that are no large changes in the relative contribution of thymic generation versus proliferation to the T-cell compartment of tonsillar cells in PFAPA patients.

3.3. B lymphocyte subsets

The proportion of CD19^{pos} B lymphocytes was lower in the PFAPA tonsils than in the controls (median 54.4% in PFAPA vs. 65.9% in controls, $p=0.02$, Fig. 1A and Supplementary Fig. 2A). There was a significantly higher proportion of CD19^{pos}CD27^{neg}CD24^{high}CD38^{high} transitional B cells in the PFAPA tonsils, yet the numbers of those cells in both the PFAPA and control groups were extremely low (median 0.5% of CD19^{pos} cells in PFAPA vs. 0.1% in controls, $p=0.035$, Table 1). There was no difference in the proportion of naïve CD27^{neg} B lymphocytes in the PFAPA tonsils compared with that of the controls (Table 1). The percentage of switched B lymphocytes defined as CD19^{pos}IgM^{neg}IgD^{neg} (Supplementary Fig. 2C) among CD19^{pos} B cells was significantly lower in the PFAPA tonsils (median 38% among CD19^{pos} in PFAPA vs. median 54% in controls, $p=0.035$, Table 1 and median 22% from

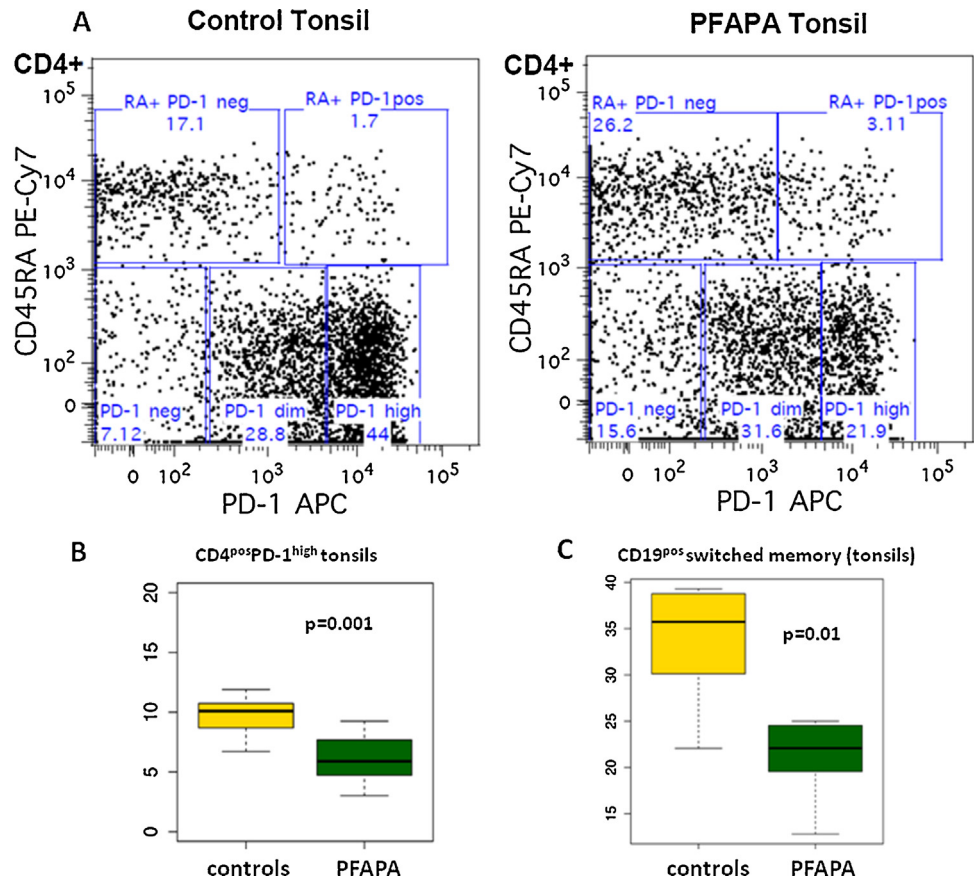


Fig. 2. PD-1 expression in PFAPA vs. controls in tonsils. (A) Flow cytometry evaluation of the CD4^{pos} cells in the tonsils of patients with PFAPA and in control tonsils. (B) The percentage of CD4^{pos}PD-1^{high} T lymphocytes from all lymphocytes. (C) The percentage of switched memory B cells from all lymphocytes.

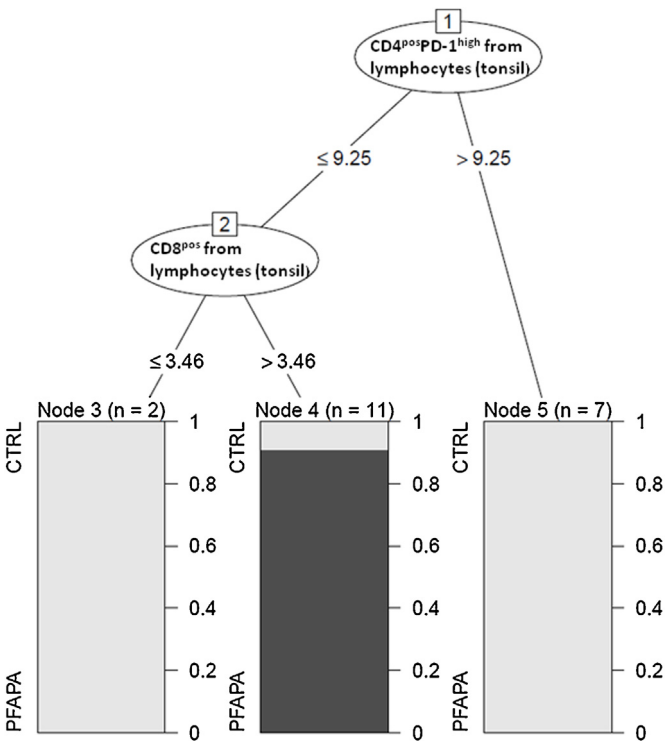


Fig. 3. Classification tree based on information from all measured T-lymphocyte populations.

all lymphocytes in PFAPA vs. 36% in controls, $p=0.01$, Fig. 2C). The lower numbers of switched B cells in PFAPA tonsils were attributed to a reduction in the CD27^{neg} switched compartment ($p=0.035$, Table 1).

Again, the observed differences were limited to tonsils and were not present in the blood (Table 1). The only exceptions were the numbers of plasma B cells (defined as CD19^{pos} CD27^{high}/CD38^{high}, Supplementary Fig. 2D), the numbers of which were lower in the PFAPA tonsils (median 1.1% of CD19^{pos} cells in PFAPA vs. 2% in controls) but which were slightly higher in the blood of patients with PFAPA (median 3%) compared with the blood of the control patients (median 1.2%, $p=0.02$). Absolute counts of all analysed B cell subpopulations did not differ between PFAPA and controls. The numbers of KREC were not significantly different in PFAPA tonsils (Supplementary Fig. 3).

3.4. Both PFAPA and control tonsils contain DNA of common viruses

Based on the higher levels of cytotoxic T lymphocytes in the PFAPA tonsils, we speculated that a common viral infection may be the trigger for T lymphocyte recruitment. Quantitative PCR for adenovirus, cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Human herpes virus 6 (HHV6) were performed. CMV was not detected in any of the samples. A low viral load of at least one of the investigated species was present in 7 of 10 of the PFAPA tonsils but also in 7 of 9 of the investigated control tonsils (Table 2). The positive detection of viruses did not differ between the PFAPA cases and controls.

Table 2

Normalised copies of viral DNA (per 10,000 copies of human albumin gene) assessed via qPCR in PFAPA and control tonsils.

Patient	EBV	CMV	HHV6	adeno
PFAPA	0	0	0	0
PFAPA	1.4	0	0	0
PFAPA	0	0	6.4	110
PFAPA	0	0	0	18
PFAPA	0	0	1.7	0
PFAPA	6.4	0	0	0
PFAPA	0	0	3.8	0
PFAPA	0	0	6	440
PFAPA	0	0	0	0
CTRL	3.3	0	0	78.1
CTRL	0	0	0	0
CTRL	8.3	0	0	0
CTRL	30	0	0	0
CTRL	1.6	0	2.4	0
CTRL	0	0	6.7	9
CTRL	0	0	0	0
CTRL	0.71	0	0	0
CTRL	0	0	1.5	0

3.5. Immunoglobulin and T-cell receptor gene rearrangement analysis reveals a polyclonal pattern in PFAPA tonsils

The patterns of the rearrangements of IGH, TCRB and TCRG were evaluated using DNA from blood and tonsils in search for a putative clonal/oligoclonal proliferation. Patients with PFAPA showed a normal Gaussian distribution of PCR fragment sizes with no traces of clonality/oligoclonality (data not shown). The PFAPA tonsils had slightly elevated numbers of gamma delta T lymphocytes. We explored the sequence motifs of multiple TCRG rearrangements generated via multiplex PCR and next generation sequencing in four PFAPA tonsils and three control tonsils aiming at finding a possible subset of gamma delta T lymphocytes shared in PFAPA and not present in controls. Interestingly, the clone CALWEVYYKLL, which was highly represented in PFAPA patient no. 3, has been previously detected in the peripheral blood of a patient with periodic fever syndrome (Jouen-Beades et al., 1999). Nevertheless, the highly sensitive NGS method detected the same clone (weakly represented, 0.001%–0.1%) in all of the PFAPA and control tonsils. Also the other public TCRG rearrangements were present in both PFAPA and controls (Supplementary Material and Supplementary Table 1).

Most of TCRG rearrangements in unsorted T lymphocytes are non-functional, accompany productive TCRB rearrangements in TCR $\alpha\beta$ lymphocytes and are present in virtually all T lymphocytes (Sherwood et al., 2011; Fronkova et al., 2005). Consistent with this, most of the TCRG rearrangements in tonsils were non-functional (unproductive or using pseudogene), apparently corresponding to TCR $\alpha\beta$ lymphocytes, and did not show any larger (>1%) clonal/oligoclonal expansion.

3.6. PFAPA tonsils have a higher expression of T cell chemoattractant genes

The expression levels of CXCL1, CXCL9, CXCL10, CXCL12, CXCL13 and CCL19 genes in PFAPA tonsils are shown in Fig. 4. The levels of CXCL9, CXCL10 and CCL19 were significantly higher in PFAPA tonsils ($p=0.0006$, $p=0.001$ and $p=0.01$, respectively). There was a similar yet not significant trend for CXCL12 ($p=0.08$). There was no difference in CXCL1 and CXCL13 gene expression.

4. Discussion

To our knowledge, our study is the first to explore in detail the lymphocyte subsets in PFAPA tonsils. A recently published study exploring the tonsils of patients with PFAPA examined mRNA

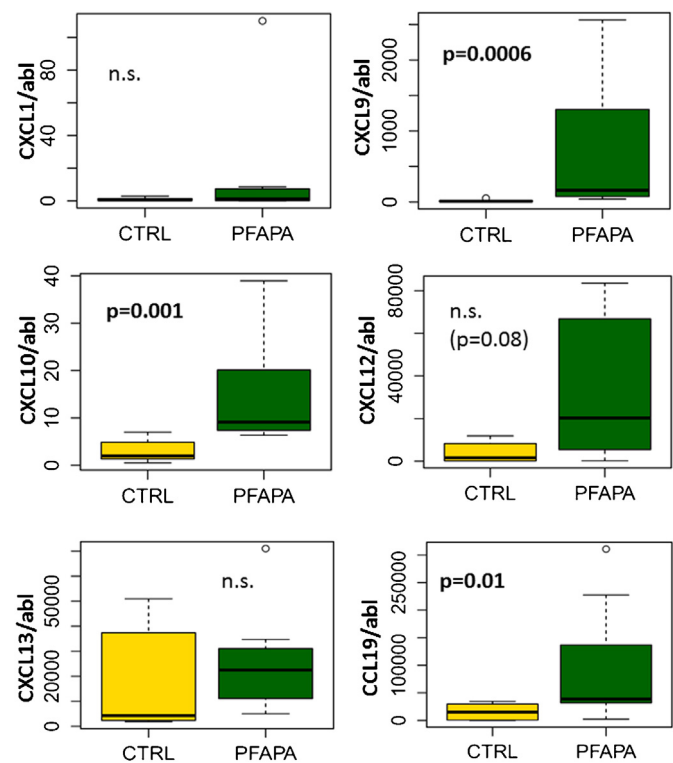


Fig. 4. The gene expression levels of CXCL1, CXCL9, CXCL10, CXCL12, CXCL13 and CCL19 genes in PFAPA tonsils are shown relatively to the expression levels in mixed peripheral blood of five healthy donors.

expression of various cytokines and found IL-1 β , TNF- α , TGF- β , IL-17 and IFN- γ mRNA levels that were similar to control hypertrophic tonsils, with the only exception of IL-4 being lower in the PFAPA tonsils (Valenzuela et al., 2013). Chen et al. (Chen et al., 2009) investigated the tissue sections of patients with autoinflammatory syndromes including one tonsil from a patient with PFAPA and found that the tonsil of the PFAPA patient contained more basophils with bound or internalised IgD. Mucosal basophils in autoinflammatory syndromes could enhance inflammation by releasing IL-1 β and LL-37 in response to microbial antigens with IgD-binding activity. The plasma IgD^{pos}IgM^{neg} class switched cells were elevated in both the blood and tonsils of patients with autoinflammatory syndromes. In our study, we found increased numbers of plasma cells in the peripheral blood of PFAPA patients. However, we could not assess their IgD production, as we only measured the surface (not intracellular) IgD in our study. IgD isolated from human serum has been demonstrated to enhance the release of IL-1 β , TNF- α and interleukin-1 receptor antagonist from human mononuclear cells (Drenth et al., 1996). A recent study suggests that monocytes trigger the autoinflammatory cascade through the release of IL-1 β (Kolly et al., 2013). In the experimental setting of our study, we could only indirectly assess the numbers of monocytes, which did not differ between the PFAPA and control tonsils (data not shown).

In our study, we observed elevated levels of the naïve stages of both CD4 and CD8T cells and an approximately 2-fold increase in the CD8T cell proportion in the PFAPA tonsils. Because the TREC levels were the same or even higher in the PFAPA tonsils, the increased numbers of T lymphocytes were not due to homeostatic proliferation but instead corresponded to newly arrived cells. All investigated parameters were concordant among the tonsil pairs in our patients; the pathogenetic mechanism apparently affects the tonsils as a whole instead of through an isolated focal process. A study by Brown et al. (Brown et al., 2010) speculated that reduced concentrations of IL-7, IL-17 and IFN γ in the afebrile interval may

indicate defective T cell regulation in PFAPA syndrome. We found an approximately 2-fold lower percentage of CD4^{pos} T cells with a high expression of the inhibitory molecule PD-1 in the PFAPA tonsils. CD4^{pos} T cells have been described to be localised in the rim of germinal centres and to have the main primary function of helping B cells, mostly via producing various factors including LFA3, CD40L, ICOS, OX40, and CD10. Neutralising antibodies against CD40L or ICOS abolished the increased antibody production by B cells cocultivated with CD4^{pos}PD-1^{high} T cells (Wang et al., 2011). Consistent with this finding, we found a decreased number of switched memory B cells in the PFAPA tonsils, suggesting that variations in the T cell compartment cause perturbations in the B cell functions in tonsils. Three studies (Brown et al., 2010; Stojanov et al., 2011; Forsvoll et al., 2013) found increased levels of chemokines that are known T-cell chemoattractants during PFAPA flares compared with asymptomatic intervals. The levels of CXCL10 protein remained increased in afebrile period in the plasma in one study (Forsvoll et al., 2013), while CXCL9 protein was found increased, yet lower than during PFAPA flares, in another study (Stojanov et al., 2011). The numbers of both the CD4 and CD8T cells in PB were reduced during PFAPA flares (Stojanov et al., 2011). This could indicate that the elevated numbers of CD8T cells in tonsils taken in asymptomatic periods in our study originate from their recruitment to tonsils from blood during PFAPA flares. We found significantly higher levels of CXCL9, CXCL10 and CCL19 gene expression in PFAPA tonsils even in afebrile period. All three proteins participate in T cell chemoattraction. CXCL1, a chemokine with neutrophil chemoattractant activity, was found increased in PB during PFAPA flares, but the gene expression was normal in afebrile period (Stojanov et al., 2011). Consistent with this, we found no difference in tonsillar CXCL1 expression between attacks. The gene expression levels of CXCL13, a known chemoattractant for B lymphocytes, did not differ in PFAPA tonsils. This further supports our findings that B cells are not as important as T cells in PFAPA pathogenesis.

A study by Stojanov et al. (Stojanov et al., 2011) found slightly elevated levels of gamma delta T lymphocytes during the asymptomatic period. Using the numbers of CD3^{high}DN cells, we set the approximate value of gamma delta T cells to be slightly elevated in the PFAPA tonsils. However, the compartment of CD3^{high}DN cells does not contain solely gamma delta T cells. A T cell receptor gamma with a CALWEVYYKKL CDR3 amino acid sequence was previously described in a patient with recurrent fever and was abundant in tonsil of one PFAPA patient (Jouen-Beades et al., 1999), but it was also present in all remaining PFAPA and control tonsils. Additionally, the spectrum of other TCRG rearrangements overlapped among the PFAPA and controls. Thus, the gamma delta T cells respond to some unspecific stimuli rather than a specific antigen in PFAPA patients. The composition of the lymphocyte subsets with an elevation of CD8 cells in our study raised the question of whether a viral antigen could be a trigger of PFAPA flares. There have been no studies examining viral loads in PFAPA patients thus far. We found DNA from EBV, HHV-6 and adenovirus but not cytomegalovirus in most PFAPA and also in most control tonsils. Very recently, a study exploring transcriptome of 6 PFAPA tonsils and 8 controls in search of infectious triggers of PFAPA has found human coxsackievirus, parechovirus and adenovirus C RNA at low abundance in a subset of samples but again without enrichment of any of these viruses in PFAPA cases (Freeman et al., *In press*). Therefore, a virus as a potential trigger of PFAPA flare is not excluded; however, other cooperating factors are probably necessary. Two recent studies found variants in inflammasome-related genes in a part of PFAPA patients (Kolly et al., 2013 Jun; Kubota et al., 2014), which supports the hypothesis of a dysregulated immune response to a yet unidentified trigger.

The tonsillectomies have been curative in all of the patients in our PFAPA group, a result that is similar to other published

reports (Feder and Salazar, 2010; Garavello et al., 2009; Peridis et al., 2010a). There were no further episodes of PFAPA after tonsillectomy in these patients over an 8-month follow up period. Our case series is similar to others in the literature regarding most frequent clinical symptoms (Feder and Salazar, 2010 Feb; Marshall et al., 1987; Tasher et al., 2006). A recurrence of aphthous stomatitis in PFAPA patients after surgery has been previously described in some studies (Valenzuela et al., 2013; Pignataro et al., 2009; Vigo et al., 2014), but this was not a finding of our study. The median age at surgery in PFAPA patients was also similar to that reported in other series (Feder and Salazar, 2010; Valenzuela et al., 2013). In line with previously published findings, the histology results of all of the PFAPA tonsils corresponded with the signs of chronic tonsillar inflammation, and the PFAPA tonsils cultivation examinations were negative for bacteria in all patients (Peridis et al., 2010b; Pignataro et al., 2009). The optimal treatment of PFAPA syndrome remains a matter of a debate. The administration of antibiotics and non-steroidal anti-inflammatory agents has been judged to be ineffective (Thomas et al., 1999). The most effective non-surgical treatment is episodic administration of a corticosteroid with positive clinical effects in 80–90% of the patients (Feder and Salazar, 2010; Berlucchi et al., 2003; Wurster et al., 2011; Krol et al., 2013). Nevertheless, a single dose of prednisone administered at the febrile episode onset does not prevent the recurrence of fevers. The shortening of afebrile intervals in up to 50% of prednisone-treated patients was also described in some studies (Feder and Salazar, 2010). The use of additional prednisone doses in cases of insufficient response has been reported by some authors (Thomas et al., 1999; Tasher et al., 2006); however, other authors did not recommend this method without prior medical reassessment to exclude infection (Krol et al., 2013). The recently published meta-analysis reported equal effects of corticotherapy and tonsillectomy, but tonsillectomy (+/– adenoidectomy) was the most effective intervention for the long-term resolution of PFAPA syndrome (Peridis et al., 2010a). There is no apparent difference in patient outcome regarding whether an adenoidectomy is performed alongside the tonsillectomy (Wong et al., 2008). In a recent study (Stojanov et al., 2011), five PFAPA patients were treated with the recombinant IL-1 receptor antagonist anakinra with a prompt clinical response. According to the literature, PFAPA syndrome resolves spontaneously after several years; the study with the longest follow up described disease remission in 50/59 patients whose disease lasted for a mean of 6.3 years (Wurster et al., 2011).

5. Conclusion

A positive effect of tonsillectomy was proven in 100% of the PFAPA patients in our study. Most of the observed differences comprised T lymphocyte compartments and were limited to tonsils. This can partially explain the mechanism for therapeutic success in tonsillectomy. The increased numbers of polyclonal and early developmental stages of T lymphocytes accompanied by an elevation in gene expression of T cell chemoattractants in PFAPA tonsils suggest their recruitment from the PB to the tonsils via impaired chemokine expression in PFAPA.

Acknowledgements

This work was supported by GAUK 266411. T.K. and J.T. were supported by the GACR Centre of Excellence P302/12/G101. E.F. received the LiOreal for Women in Science Fellowship in 2013. M.K. was supported by IGA NT/14343-3. We thank Jan Stuchly for help with the data analysis. The authors are most grateful to doc. Zdeněk Kabelka, Ph. D. (passed away on 24 March 2014) for his expertise

and invaluable help with all aspects of the study. The authors wish to dedicate this paper to his memory.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2015.01.004>.

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RESEARCH ARTICLE

The TREC/KREC Assay for the Diagnosis and Monitoring of Patients with DiGeorge Syndrome

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Citation: Froňková E, Klocperk A, Svatoň M, Nováková M, Kotrová M, et al. (2014) The TREC/KREC Assay for the Diagnosis and Monitoring of Patients with DiGeorge Syndrome. PLoS ONE 9(12): e114514. doi:10.1371/journal.pone.0114514

Editor: Matthaios Speletas, University of Thessaly, Faculty of Medicine, Greece

Received: June 4, 2014

Accepted: November 10, 2014

Published: December 8, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: Support was provided by the Czech Science Foundation (GACR) Centre of Excellence, P302/12/G101, <http://www.gacr.cz/en/>, (EF, JT); Internal Grant Agency of the Ministry of Health of the Czech Republic, IGA MZCR NT/13287-4/2012, <http://iga.mzcr.cz/publicWeb/> (AS); Ministry of Health of the Czech Republic (MH CZ-DRO), University Hospital Motol, Prague, Czech Republic, 00064203, <http://www.mzcr.cz/> (AS); L'Oreal for Women in Science Fellowship in 2013, <http://www.prozenyvevede.cz/> (EF); Science Development Program at Charles University in Prague, PRVOUK P31, <http://ips.fsv.cuni.cz/IPSENG-53.html> (FV). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Abstract

DiGeorge syndrome (DGS) presents with a wide spectrum of thymic pathologies. Nationwide neonatal screening programs of lymphocyte production using T-cell recombination excision circles (TREC) have repeatedly identified patients with DGS. We tested what proportion of DGS patients could be identified at birth by combined TREC and kappa-deleting element recombination circle (KREC) screening. Furthermore, we followed TREC/KREC levels in peripheral blood (PB) to monitor postnatal changes in lymphocyte production.

Methods: TREC/KREC copies were assessed by quantitative PCR (qPCR) and were related to the albumin control gene in dry blood spots (DBSs) from control (n=56), severe immunodeficiency syndrome (SCID, n=10) and DGS (n=13) newborns. PB was evaluated in DGS children (n=32), in diagnostic samples from SCID babies (n=5) and in 91 controls.

Results: All but one DGS patient had TREC levels in the normal range at birth, albeit quantitative TREC values were significantly lower in the DGS cohort. One patient had slightly reduced KREC at birth. Postnatal DGS samples revealed reduced TREC numbers in 5 of 32 (16%) patients, whereas KREC copy numbers were similar to controls. Both TREC and KREC levels showed a more pronounced decrease with age in DGS patients than in controls ($p < 0.0001$ for both in a linear model). DGS patients had higher percentages of NK cells at the expense of T cells

($p < 0.0001$). The patients with reduced TREC levels had repeated infections in infancy and developed allergy and/or autoimmunity, but they were not strikingly different from other patients. In 12 DGS patients with paired DBS and blood samples, the TREC/KREC levels were mostly stable or increased and showed similar kinetics in respective patients.

Conclusions: The combined TREC/KREC approach with correction via control gene identified 1 of 13 (8%) of DiGeorge syndrome patients at birth in our cohort. The majority of patients had TREC/KREC levels in the normal range.

Introduction

DiGeorge syndrome is a complex disorder caused by an embryopathy; it presents with a number of clinical symptoms arising from the disturbed development of the 3rd and 4th pharyngeal arches, chief amongst which are congenital heart, great vessel and parathyroid gland defects as well as a typical malformation of the face and soft palate. The embryopathy is most commonly caused by a deletion on the 22nd chromosome, called del22q11, but can be caused by other mutations as well [1]. The unifying sign of DiGeorge syndrome is a wide spectrum of pathologies of the thymus, ranging from complete athymia, found in complete DiGeorge syndrome patients, to various degrees of thymic dysplasia and disturbed migration of the thymus observed in partial DiGeorge syndrome patients [2].

The degree of thymic pathology correlates with various degrees of T-lymphocytic dysfunction [3]. The thymus is the location of the T-lymphocyte maturation and selection process. In children, it is a dominant place of T-lymphocyte development and a source of naïve T cells [4]. The number of naïve T-lymphocytes that went through the thymic maturation process can be assessed from peripheral blood using T cell receptor excision circle (TREC) detection by quantitative PCR [5]. In DiGeorge syndrome, the immunodeficiency is caused by dysplasia of the thymus, and it would therefore be logical to expect abnormal development of T-cells in the thymus and accordingly abnormal TREC levels. This assumption was partially proven to be correct with the introduction of SCID (severe combined immune deficiency) screening, performed during the last 2 years in an increasing number of countries, the USA in particular [6, 7]. Apart from great sensitivity in detecting SCID patients, this screening program repeatedly detected children with DiGeorge syndrome, as previously published [6, 7]. However, the population data suggest that only a minority of DiGeorge syndrome patients with the most severe T-lymphopenia can be identified via TREC screening. Analogous to T lymphocytes, newly developed B cells can be detected using kappa-deleting element recombination circles (KREC) [8]. A combined TREC/KREC approach has been able to identify patients with severe B cell disorders such as X-linked agammaglobulinemia in addition to SCID patients [9]. Various disorders in B cell function, supposedly originating from disturbed T

and B cell interactions, have been described [1, 10]. In our study, we performed retrospective combined TREC/KREC monitoring with correction for control gene amplification in a cohort of patients with DiGeorge syndrome, and it enabled us to directly compare the results from neonatal dry blood spots with levels in peripheral blood postnatally. We aimed at finding out the percentage of DiGeorge syndrome patients who could be identified via TREC screening of newborns using our methodology. Furthermore, we tested whether addition of KREC could add more information both at birth and during the postnatal period in DiGeorge syndrome patients.

Patients and Methods

Patients

Thirty-two children (age 1.5–18 years) with DiGeorge syndrome with verified deletion of 22q11 and macroscopically missing/dysplastic/hypoplastic thymus were investigated. Del22q11.2 was verified either via FISH (fluorescent in-situ hybridisation) using the DiGeorge/VCFS TUPLE 1/22q Deletion Syndrome LPU004 probe (Cytocell, Cambridge, UK) or by using Salsa MLPA (Multiplex Ligation Probe Amplification) kits P-245-B1 or P-250-2B (MRC Holland). Twenty-eight of the 32 DGS patients underwent cardiac surgery. In 10 patients the thymus was reported as missing in the surgery protocol; in 4 patients the thymus was found to be hypoplastic and was subtotally resected; and in 3 patients only subtotal thymus resection was reported. In 10 patients, there was no information about thymic appearance; however, most of the remaining tissue was presumably also resected during surgery. Four patients had no surgery and were investigated and genetically verified based on their clinical symptoms. As controls, residual blood samples after routine clinical evaluations due to medical conditions that do not affect the lymphocyte count were used, or samples from healthy siblings of patients investigated at our department were used providing that they signed a written informed consent for the use of residual material (in total 91 children aged 1–18 years). Samples from five consecutively diagnosed patients with SCID in 2011–2013 in the Czech Republic (age of 2–4 months) were also evaluated. Archived neonatal DBSs were examined for 13 patients with DiGeorge syndrome and 10 patients with SCID. All patients or their parents provided written informed consent for the use of residual PB or DBSs in accordance with the Declaration of Helsinki. Fifty-six control DBSs were randomly chosen as controls, with an attempt to cover a DBS storage period similar to those of the patients. The birth weight of control newborns was recorded and subsequently the control DBSs were anonymised. The study was approved by the Ethics Committee of 2nd Medical School in Prague, Czech Republic.

DNA isolation

DNA was isolated from 200 µl of peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). From each DBS, a circle with a diameter of 3.2 µm was cut and DNA was eluted at 99°C for 1 hour in a shaker (500 rpm) using 100 µl of Generation DNA Elution Solution (Qiagen GmbH, Hilden, Germany) supplemented with 100 µg/ml of yeast tRNA (Life Technologies, Carlsbad, CA, USA).

TREC and KREC detection

The albumin gene level was quantitatively detected in isolated samples using qPCR [11] and a standard dilution series derived from human genomic DNA with a known starting concentration (Roche, Basel, Switzerland). The levels of TREC and KREC signal joints were assessed separately using serial dilutions of cloned plasmid standards as previously described [8, 12]. One microlitre of DNA was added to each reaction. The results were subsequently recalculated to the levels of the albumin gene and expressed as the number of TREC (KREC) copies per one microgram of DNA.

Statistical analysis

The distribution of variables between groups was assessed using the chi-squared or Fisher's exact test. The Mann-Whitney test was used to estimate the significance of the differences in continuous values. These statistical analyses were performed using StatView version 5.0 (StatView Software, Cary, NC). For evaluating TREC (KREC) dependence on age in patients and controls, a linear model was developed using R-package 3.1.1. (www.r-project.org).

Results

The levels of TREC/KREC in Guthrie cards

The storage time of the DBSs was 1–18 years. The analysis of control gene amplification in DGS and SCID patients showed no difference between long- and short-term stored DBS (data not shown). First, we established ranges for normal and abnormal TREC and KREC levels. Most current approaches use either TREC alone or combined TREC/KREC detection without correction for DNA quality. Therefore, the numerical TREC/KREC values are not directly comparable between patients. We adopted an approach presented by Kamae et al [13]. Here, TREC/KREC copy numbers are expressed per one microgram of DNA, the DNA concentration being assessed by qPCR. We assessed TREC/KREC values in DBSs of 56 control newborns and in 10 patients with SCID. Three SCID patients were both TREC- and KREC-negative (2 × RAG1 deficiency, 1 × ADA deficiency). The remaining seven patients (6 × common gamma chain mutation, 1 × unknown mutation) had negative TRECs while their KREC levels were similar to controls (

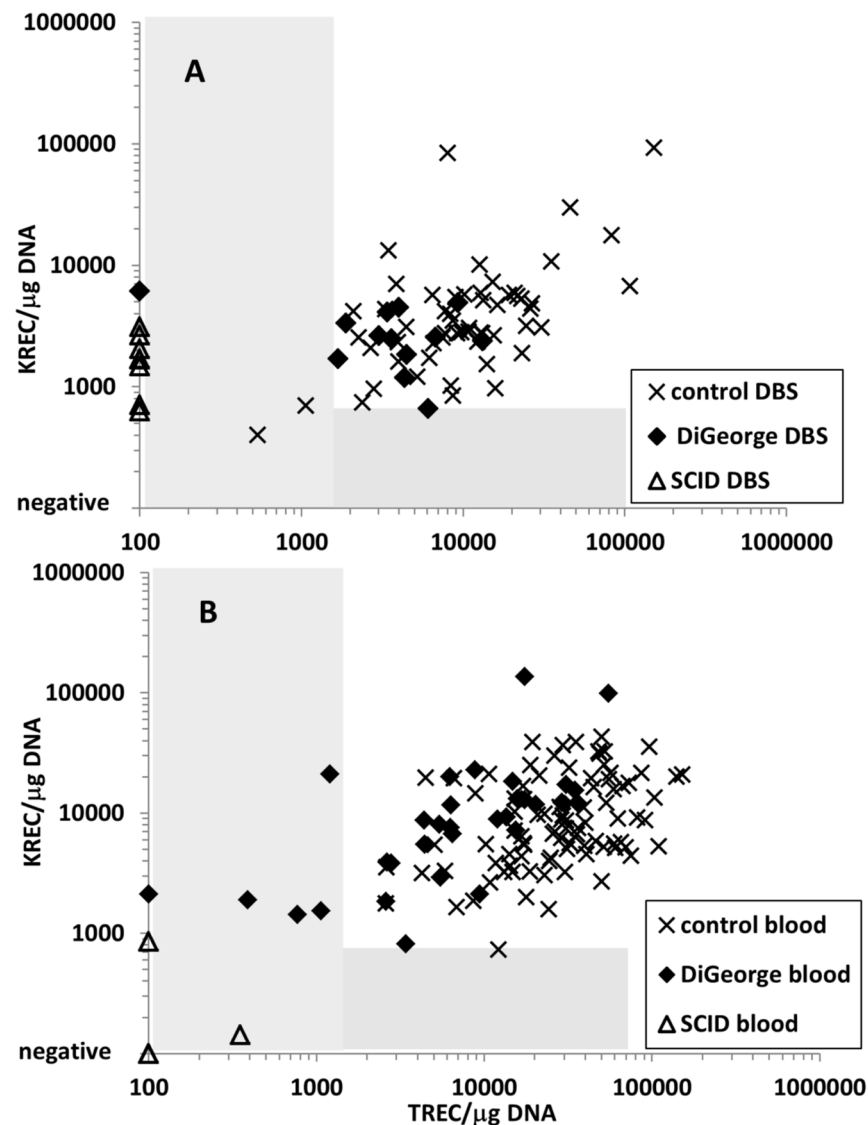


Figure 1. TREC and KREC numbers expressed as copies per microgram of DNA in A) neonatal dry blood spots B) peripheral blood in children (0–18 years) for controls, patients with DiGeorge syndrome and patients with severe combined immunodeficiency (SCID). Grey areas represent the abnormal TREC/KREC range.

doi:10.1371/journal.pone.0114514.g001

Figure 1A). Based on those findings, we set the cut-offs at 700 copies/μg DNA for KREC and 1400 copies/μg DNA for TREC. Of note, the control DBS with the lowest TREC/KREC values outside the determined range was from a premature child with birth weight of 890 grams. Figure S1 shows the same values copy numbers per one μl of dried blood without normalisation to a control gene.

Of the patients with DiGeorge syndrome, only one had completely negative TREC levels with normal KREC levels. This patient with complete athymia and the clinical picture of CHARGE syndrome received two DLIs from an unrelated

donor at the age of six months [14]. Now, at the age of 9.5, the patient has reduced numbers of T lymphocytes with no naïve T cells, but suffers from no major infection complications. However, the TREC levels in his PB remain negative. The remaining twelve patients with DiGeorge syndrome had TREC levels within the normal range, and all but one also had normal KREC levels. One patient had KREC levels near the cut-off (664 copies/μg DNA). Quantitative TREC values were significantly lower in DiGeorge syndrome DBSs (Figure 2A), even after exclusion of the TREC-negative patient from the analysis (TREC: median 4181 in DiGeorge vs. 9924 copies/μg DNA in controls, $p=0.004$, KREC: median 2552 in DiGeorge vs. 3156 in controls, n.s., Mann-Whitney).

Postnatal TREC/KREC values and their development in children with DiGeorge syndrome

The monitoring of TREC/KREC postnatally is used in research studies, but it is not routinely used in clinical examination of patients with immunodeficiency. We collected TREC/KREC values in a cohort of 91 control PB samples from children aged 0–18 years. Although there is a published trend of both TREC and KREC decrease postnatally [13, 15, 16], our data from healthy children show that KREC levels declined only slightly with age (test in linear model, $p=0.015$, Figure 3B), whereas TREC levels remained practically stable until the age of 18 ($p=0.33$, Figure 3A). More controls from different age groups would be necessary to precisely define the ranges for the respective KREC cut-offs. We therefore retained the KREC cut-offs for abnormal findings obtained from DBSs, but the declining trend has to be kept in mind. The absolute lymphocyte counts and absolute and relative B cell counts were not significantly different between DGS syndrome patients and controls (Figure 3G, F, D). As expected, the absolute and relative numbers of T lymphocytes were significantly lower in DGS patients than in controls ($p=0.03$ and $p=0.0003$, Mann-Whitney, Figure 3E, C). The percentages of NK cells were significantly higher in DGS syndrome patients ($p<0.0001$, Mann-Whitney, Figure 3H).

In the group of 32 children with DiGeorge syndrome aged 1.5 to 18 years, the TREC values were under the normal range in 5 of 32 (16%) patients (Figure 1B). Even after excluding the complete DiGeorge syndrome patient after DLI with continuously negative TREC levels, the quantitative TREC values remained significantly lower than in controls (median 6444 copies/μg DNA in DiGeorge syndrome vs. 29343 copies/μg DNA in controls, $p<0.0001$, Figure 2B). Interestingly, unlike in controls, the TREC levels declined significantly with age in DiGeorge syndrome patients (linear model, $p<0.0001$). The KREC levels were not significantly different from those of the controls (n.s., Figures 1B and 2B). However, the KREC decline during childhood was more pronounced in patients (test in linear model, $p<0.0001$) than in controls ($p=0.0047$, Figure 3B).

The four patients with TREC values under the normal range had repeated respiratory infections in infancy. All of them later developed allergies and/or autoimmunity in a form of autoimmune thyroiditis or thrombocytopenia,

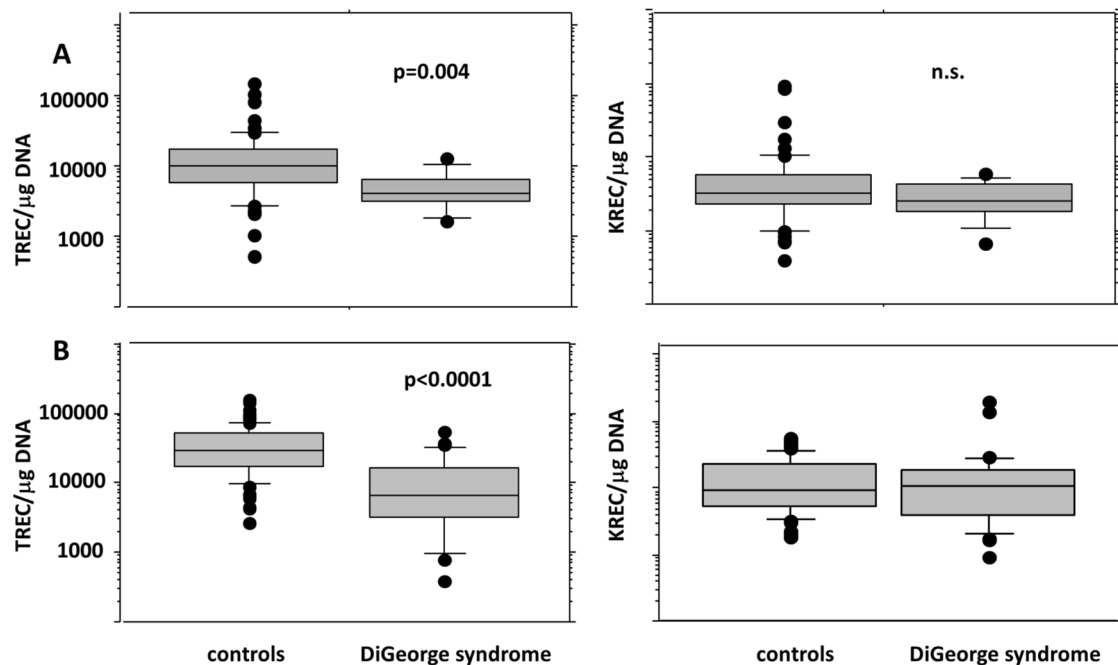


Figure 2. Quantitative TREC/KREC levels expressed as number of copies per microgram of DNA in A) neonatal dry blood spots B) peripheral blood for control children vs. children with DiGeorge syndrome (0–18 years).

doi:10.1371/journal.pone.0114514.g002

autoimmune conditions typically associated with DiGeorge syndrome. Their status, however, is not markedly different from that of other patients with DiGeorge syndrome.

In 12 patients, it was possible to monitor TREC/KREC development from birth to the present. [Figure S2A](#) shows that TREC levels remained stable (less than 10% difference, $n=4$) or increased (1.4–9-fold, $n=6$) in 10 of 12 (80%) patients. Similarly, the KREC levels were stable or increased in 75% of patients ([Figure S2B](#)). Thus, based on these data, no marked deterioration in postnatal lymphocyte output occurs in DiGeorge syndrome patients. Interestingly, the trend was consistent between TREC and KREC. The patients with a decrease in TREC had lower or stable KREC numbers, and vice versa.

Feasibility of TREC/KREC analysis in postnatal peripheral blood for differential diagnostics of primary immunodeficiencies

Neonatal screening for SCID is not yet performed in the Czech Republic. During the period of this study, we investigated five patients with suspected SCID (age 2–4 months) based on clinical findings and flow cytometry results. The TREC/KREC analysis confirmed the SCID diagnosis in those patients ([Figure 1B](#)) within the time frame of one day, and preparations for transplantation of hematopoietic stem cells started while exploring the molecular genetic cause of the SCID.

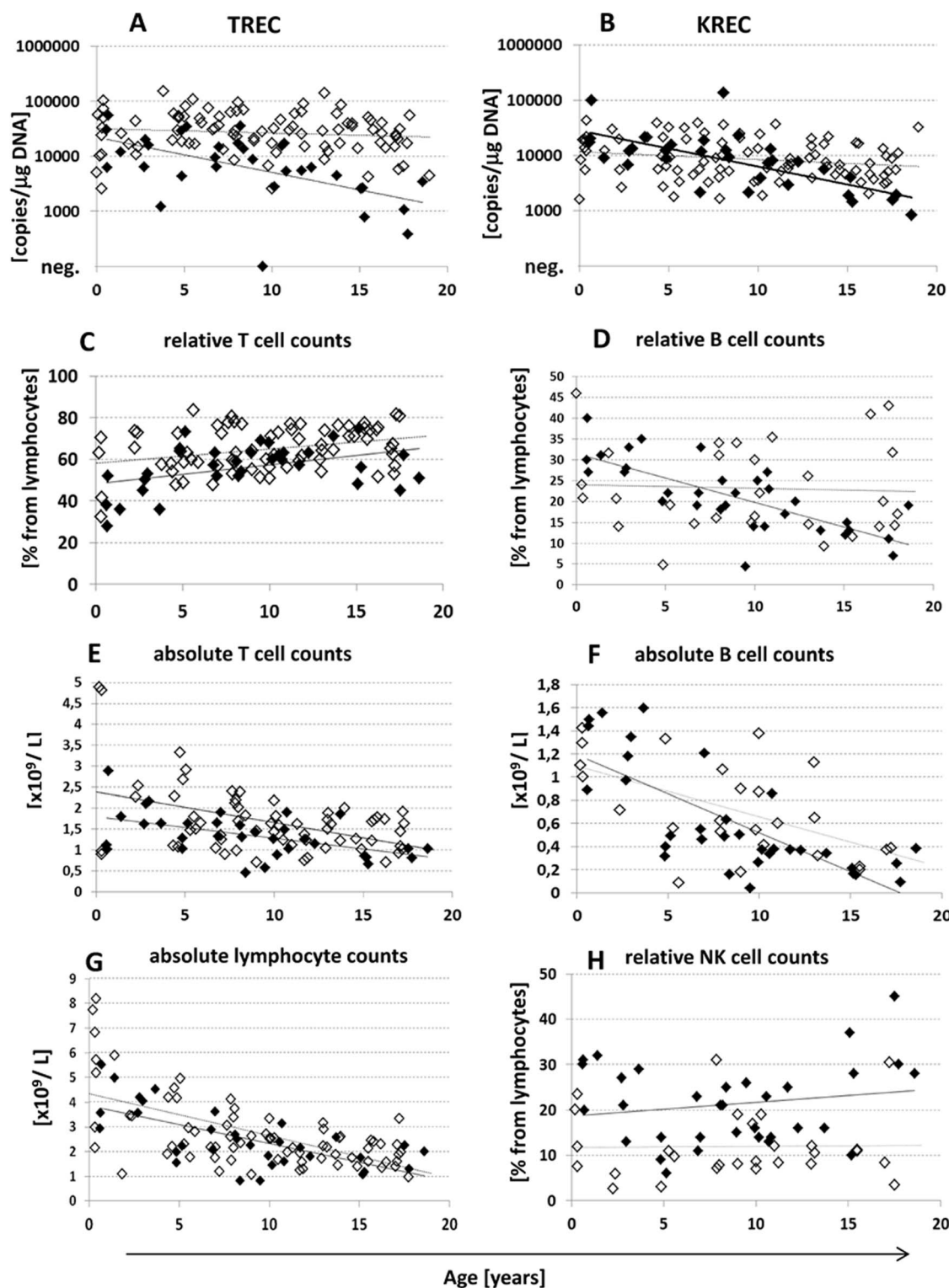


Figure 3. Quantitative levels of TRECs (A) KRECs (B), relative T cell (CD3^{pos}) counts (C), relative B cell (CD19^{pos}) counts (D), absolute T cell counts (E), absolute B cell counts (F), absolute lymphocyte counts (G) and relative NK cell (CD3^{neg}CD16^{pos}CD56^{pos}) counts (H) in relation to age (x-axis) in patients with DiGeorge syndrome (black diamonds) and in controls (empty diamonds). Lines show linear regression analyses for patients (full lines) and controls (dotted lines).

doi:10.1371/journal.pone.0114514.g003

Discussion

In the retrospective part of our study we measured TRECs in newborn dry blood spots from patients with clinically diagnosed and genetically verified DiGeorge syndrome. However, we were only successful in using this method to detect DiGeorge syndrome in a patient with complete athymia and the clinical picture of CHARGE syndrome [14]. All the other patients with partial DiGeorge syndrome exhibited TREC levels within the normal range at newborn age. Of 993,724 infants examined in California newborn screening, only one complete and eight incomplete DiGeorge syndrome patients were identified. Given the incidence of the syndrome, the authors concluded that approximately 5% of the DiGeorge syndrome patients with the most severe T lymphopenia could be identified at birth via TREC screening [6]. Recently, a study similar to ours has been published using TREC/KREC detection without correction using a control gene [17]. The percentage of DiGeorge syndrome patients identified by neonatal screening was 19% in that study. We could only identify 8% of patients using our approach. This result may be skewed due to the low number of examined cases. Nevertheless, the data in both studies suggest that the more sensitive approach of the Swedish group also identified patients with less severe T-lymphopenia who did not require immune-restoring therapy despite suffering from severe and recurrent viral infections. This demonstrates the importance of cut-offs for reporting abnormal results, which should be widely discussed before the implementation of routine newborn screening programs.

Decreased T-lymphocyte counts during the critical period of the first few years of age in DiGeorge syndrome have been demonstrated in previously published studies [18]. We examined TRECs in patients from similar age groups. TRECs in DiGeorge syndrome have been previously investigated [15, 19, 20], and consistent with expectations they were found to be lower compared to controls. Another trend of gradual TREC decline with age was documented and published [15], which can possibly be explained by a gradual decrease in the importance of the thymus as a source of naïve T-lymphocytes as it is replaced by the division of T-lymphocytes [21]. However, our results are only partly consistent with the previously published data. In the control children, the TREC levels remained stable instead of declining during infancy (until 18 years), and we only observed a significant decrease of TREC levels with age in DGS patients. In our cohort we identified four patients with reduced TREC levels during childhood (13%), and they had repeated respiratory infections in infancy and later in life developed allergies and/or autoimmunity typical for DGS syndrome patients, but generally they did well without immune intervention and did not differ significantly from patients with normal TREC levels. Unfortunately, the Guthrie cards were not available for those patients. In the majority of DiGeorge syndrome patients, we observed no marked deviation from normal values either at birth or postnatally, even if the overall quantitative TREC values were significantly lower in both groups than in controls.

Our method uses correction for albumin gene levels for all the patients in the first-line test, which is different from most nationwide screening programs. This approach is more expensive (estimated costs ~12 USD per sample) than the “TREC-only” approach with estimated cost of 4.22 USD per sample. Recently, a newborn SCID screening study was performed by a British group using the commercial TREC EnLite kit (Perkin Elmer), and it successfully detected all SCID cases in DBS [22]. The kit does not require the isolation of DNA, which is very convenient for nationwide screening due to ethical issues. In conclusion, our method is more suitable for research studies and postnatal monitoring than for nationwide screening, having the advantage of a more precise comparison between patients and controls and allowing the monitoring of TREC/KREC development postnatally. Comparing the levels between birth and the postnatal period, we observed a 1.4 to 9-fold increase in TREC levels in 50% of the patients. This is consistent with published data on CD3-positive cell development and with clinical observations in those patients, both showing improvements after the first years of life [18]. We can speculate that extrathymic tissue, namely tonsils as recently published [23], contributed to the generation of new T-lymphocytes in those patients because in most of the children the residual thymus were destroyed during the cardiac surgery.

KREC levels were normal in most patients at birth, which is consistent with a previous report [17]. Postnatal KREC levels in patients with DiGeorge syndrome have not been studied to date. An interesting observation of our study was quantitatively lower KREC levels in one patient at birth, which were found to be normalised at the age of 12 years (showing an 11-fold increase compared to birth). This is consistent with a previous study showing lower B cell numbers in the early years of life normalising later in some patients [24].

One of the major achievements of our study was establishing a robust platform for TREC/KREC testing not only in newborn screening but also for rapid postnatal differential diagnostics of severe immunodeficiency. One has to keep in mind that normalised TREC/KREC numbers in postnatal blood can be skewed in cases with clonal expansion of mature lymphocytes, e.g., in response to infections. Indeed, the TREC/KREC levels did not correlate well with relative T (B) lymphocyte counts (Figure S3A, B). We already showed the usefulness of normalised KREC monitoring as a surrogate marker in the monitoring of B cell reconstitution after stem cell transplantation [25], and we are convinced that despite its limitations, it is a very useful independent marker showing clearly whether B/T lymphopoiesis is retained. As the nation-wide neonatal screening of SCID is not planned in the Czech Republic at least for the next year, we continue to build a network of informed paediatricians and immunologists who should ensure that all infants with a suspect clinical picture and/or family history undergo rapid TREC/KREC screening.

Conclusions

In our study, TREC levels only identified one severe case with deep lymphopaenia, which corresponds to 8% of the limited DiGeorge syndrome cohort. Most children with incomplete DiGeorge syndrome had TREC/KREC levels in the normal range both at birth and postnatally. Nevertheless, the decline of both TREC and KREC levels with age was more significant in DGS patients than in controls. Thus, the assay complements routine investigations and follow-up of DiGeorge patients and might reflect age-related immune changes based on the del22q11 background.

Supporting Information

Figure S1. TREC and KREC numbers expressed as copies per one μ l of dry blood without control gene correction for controls, patients with DiGeorge syndrome and patients with severe combined immunodeficiency (SCID).

[doi:10.1371/journal.pone.0114514.s001](https://doi.org/10.1371/journal.pone.0114514.s001) (TIF)

Figure S2. Changes in TREC (A) and KREC (B) values between birth and the present in children with DiGeorge syndrome (dotted lines). The levels in control children are depicted by empty circles. Grey areas represent the abnormal TREC/KREC range.

[doi:10.1371/journal.pone.0114514.s002](https://doi.org/10.1371/journal.pone.0114514.s002) (TIF)

Figure S3. The correlation of TREC levels with relative numbers of T lymphocytes (A) and correlation of KREC levels with relative numbers of B lymphocytes (B) for DiGeorge syndrome patients (full diamonds) and controls (empty diamonds).

[doi:10.1371/journal.pone.0114514.s003](https://doi.org/10.1371/journal.pone.0114514.s003) (TIF)

Author Contributions

Conceived and designed the experiments: EF JT AS. Performed the experiments: EF MS AK MK EM PK TF JK TK MN. Analyzed the data: EF FV HV JT AS MN. Contributed reagents/materials/analysis tools: FV HV JK. Wrote the paper: EF AS.

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