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Critically Appraised Topic

Next-generation Sequencing panel for Mature Lymphoid Malignancies

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CLINICAL BOTTOM LINE

Recent efforts in using next-generation sequencing in mature lymphoid malignancies have led to the discovery of numerous genetic aberrations which can deliver clinically relevant diagnostic, prognostic and therapeutic information in these heterogeneous group of cancers. In addition to a customized NGS panel to investigate myeloid malignancies already in use in our laboratory, interest has raised to compose a customized NGS panel to investigate genetic disorders of mature lymphomas.

These disorders include DNA variants which will be analysed via Anchored Multiplex PCR-based NGS (QIAseq, QIAGEN) and sequenced on an Illumina MiSeq platform. Based on a thorough literature search, a gene panel comprising 51 genes is selected, consisting of 67,418 base pairs to be sequenced. Genomic coordinates of all coding exons of interest are collected (GRCH37/hg19), so a design of 928 primers is created to produce a QIASeq Targeted DNA custom panel.

In the first place, this lymphoid panel will be used in routine clinical practice - subsequent to completion of the verification study - in patients with mature lymphomas in whom no conclusive diagnosis can be made with current diagnostic assays. This decision will be made during multidisciplinary oncologic consult. The interpretation of the detected variants will primarily occur according to the Belgian NGS guidelines for haematological and solid tumours. The price per sample for the laboratory is €361 excluding VAT and overhead costs; €681 including VAT and overhead costs. A distinct partial reimbursement is applicable for each lymphoma entity.

CLINICAL/DIAGNOSTIC SCENARIO

Mature malignant lymphomas, historically classified as non-Hodgkin (NHL) and Hodgkin lymphomas (HL), are a heterogeneous group of cancers with more than 100,000 new cases each year in Europe. Clinically, this heterogeneity covers very indolent to highly aggressive presentations; acquires a distinct initiation and type of treatment and is characterized by a diverse response to therapy and clinical outcome (1,2). Current diagnostic assessment consists of a combination of morphological examination, immunophenotyping, immunohistochemistry, cytogenetics, fluorescence in situ hybridization (FISH) and molecular genetics (3). To date, the WHO 2017 classification of lymphoid neoplasms defines 34 distinct entities of mature B-cell neoplasms, 19 entities of mature T- and NK-cell neoplasms and two entities of Hodgkin lymphomas (4).

Besides a considerable diversity between current entities, recent efforts in using next-generation sequencing (NGS) have revealed a highly complicated genetic landscape of these tumours, sometimes even within one entity. These complex patterns of genetic aberrations have led to a better understanding of key pathways deregulated in each lymphoma subtype (2,5). Interesting, these insights have led to postulated new genetic-based classifications. For example, the entity diffuse large B-cell lymphoma not otherwise specified (DLBCL NOS) - morphologically characterized as an aggressive B-cell lymphoma, is according to the WHO 2017 classification molecularly subdivided into germinal centre B-cell (GCB) subtype and activated B-cell (ABC) subtype. However, new genetic-based taxonomies of DLBCL NOS are recently proposed by two independent groups (SCHMITZ et al., N Engl J Med 2018 and CHAPUY et al., Nat Med 2018), whose research results partially overlap. These new taxonomies divide DLBCL NOS into four respectively six subgroups based on genetic aberrations and reflect in this way distinct mechanisms of lymphomagenesis. These subgroups had different outcome after chemotherapy, so these genetic aberrations are possibly future therapeutic targets (5).

These reports highlight that genetic profiling has the potential to deliver clinically relevant information (3). Some genetic aberrations are already introduced in the WHO 2017 classification and are nowadays identified in our laboratory. For example, sequencing of BRAF V600E mutation in Hairy cell leukaemia (HCL) or MYD88 L265P in Lymphoplasmatic lymphoma (LPL) is implemented in routine diagnostic workup. In addition, screening for some mutations with therapeutic implications or prognostic impact is performed for TP53 in Chronic lymphocytic leukaemia (CLL). Patients mutated in TP53 are linked to an impaired response to fludarabine-containing regimes and are associated with a poor prognosis (6,7). On the other hand, EZH2 mutations which are a potential therapeutic drug target in Follicular Lymphoma (FL) or mutations in NOTCH1, SF3B1 and BIRC3 of which adverse prognostic implications are described in CLL (4), are not yet routinely sequenced in the diagnostic work up of lymphoma in our laboratory.

These three pillars, i.e. diagnostic impact, prognostic impact and immediate impact on treatment decisions, are the main areas of interest of using next-generation sequencing in mature lymphoid malignancies. This technique allows to screen many genes in multiple samples simultaneously, whereby a gene panel including the most frequently mutated genes can be of additional value in the diagnosis of lymphomas. Since 2017, a next-generation sequencing (NGS) panel of myeloid malignancies including 21 genes is used in daily practice in our laboratory for new diagnoses of acute myeloid leukaemia (AML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN). Besides, a panel for mutation analysis including the TP53 and IDH2 gene is used since 2019 in case of new diagnosed CLL (TP53) and in case of new diagnosed AML or MDS in patients over the age of 70 (IDH2 + TP53). A great advantage of NGS, besides the ability of multi-gene and - sample analysis, is the higher sensitivity with which low-frequency variants can be detected. TP53 microclones for example, i.e. subclones with low-allelic burden who are associated with a poor outcome too, can be detected by NGS, but not by Sanger sequencing. This finding justifies the use of NGS for TP53 analysis in routine clinical setting (8).

To date, comprehensive NGS panels for lymphoid malignancies are not yet recommended in routine clinical practice until more data from clinical trials is available to support altering clinical management based on these results (5). However, understanding the impact of somatic mutations in these cancers advances rapidly. Several sequencing panels have been suggested, although much controversy exists regarding panel design and a "universal lymphoid" NGS panel is not yet well established (1).

The purpose of this project is to compose a NGS panel of a selection of genes in which mutations are most clinically relevant. Since the selected primers are fully customized, the composition of the panel can be modified in the future. Initially, the panel will be used as an additional diagnostic tool in lymphomas which are difficult to differentiate, together with the knowledge of morphological characteristics, immunophenotyping, immunohistochemistry, chromosomal translocations and other genetic analyses. Secondly, further therapeutic and prognostic information providing mutational profiles of actionable targets will contribute clinicians to make a solid therapeutic decision in the era of personalized medicine, since numerous new inhibitors targeting key cellular pathways are being developed (1,3,9,10).

QUESTIONS

1) Which genes should be included in a next-generation sequencing panel for mature lymphoid malignancies providing diagnostic, prognostic and therapeutic information?

2) How can such panel be implemented in routine clinical practice?

SEARCH TERMS

1) MeSH Database (PubMed): MeSH term: "B cell lymphoma " "high throughput dna sequencing" "t cell lymphoma"
2) PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific): "b cell lymphoma", "t cell lymphoma", "next-generation sequencing", "lymphoma", "gene expression", "small b cell lymphoma"
3) Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane)

RELEVANT EVIDENCE/REFERENCES

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APPRAISAL

***Question 1*. Which genes should be included in a next-generation sequencing panel for mature lymphoid malignancies providing diagnostic, prognostic and therapeutic information?**

The development of a targeted NGS gene panel is a process in which multiple items need to be completed before its use in routine clinical practice can be justified. The decision which specific genes will be included is an important step, although first the purpose of this new clinical test and secondly the acceptable clinical sample types need to be determined beforehand (11).

1.1. Purpose of the panel & acceptable clinical samples

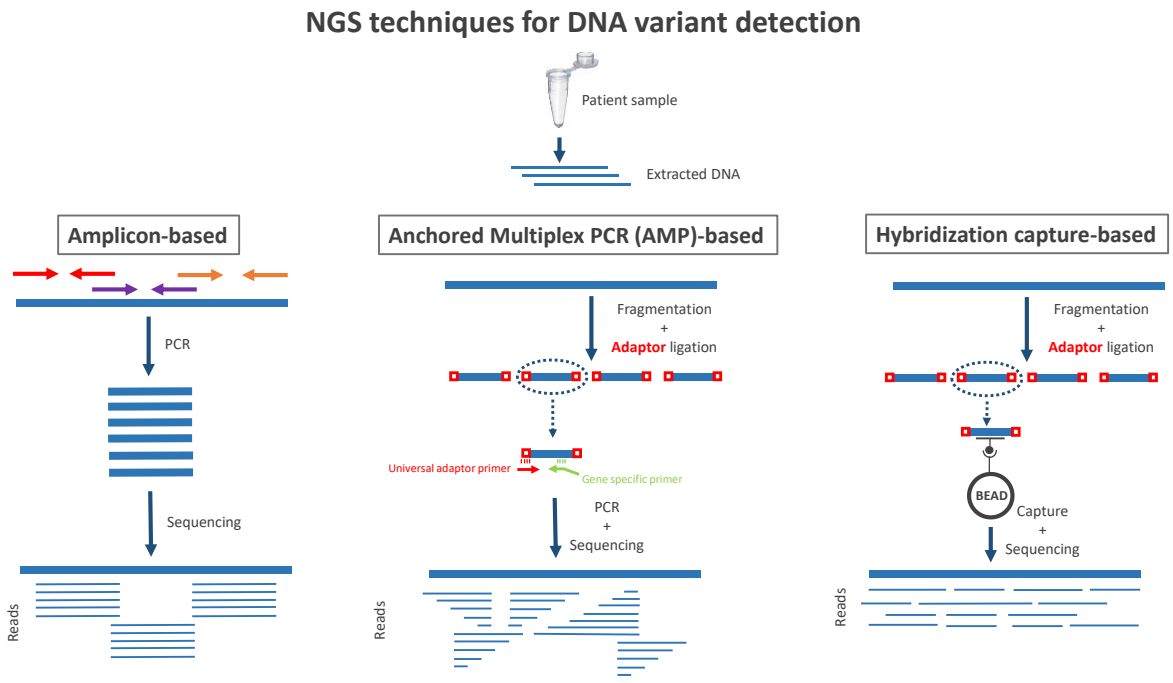
As stated in the clinical/diagnostic scenario, the purpose of this customized panel is to include those genes of which the mutations described are the most clinically relevant at this time in terms of diagnostic, prognostic and therapeutic information concerning mature lymphoid malignancies. The intended use is to include primary tumour samples, including bone marrow specimen (stored in EDTA or CPDA-1) and fresh tissue material, but no Formalin Fixed Paraffin Embedded (FFPE) samples. The reason not to include FFPE samples is because of poor quality of the DNA which requires a different design of the panel with many extra primers, resulting in an even higher cost. Whole blood samples are no first choice specimen, although this specimen type will be validated with this panel. Additionally, there is no purpose to use this panel to monitor minimal residual disease (MRD) post-therapy. The technique that is used needs to detect DNA variants, including single nucleotide variants (SNV) and small insertions and deletions (indels). No structural variants, including translocations, will be investigated. As such, knowledge of the test instrumentation is an important step before considering which genes will be included.

Hybrid capture NGS and amplification-based NGS are two major approaches commonly used for library preparation (*i.e.* the process to generate DNA of cDNA fragments of specific size range). The first method uses biotinylated oligonucleotide capture probes, which are significantly longer than PCR probes, as such avoiding allele dropout. This technique has the ability to examine large regions of the genome (from 50 genes to whole exome sequencing), in contrast to the amplification-based NGS which is more suitable for smaller gene panels. The latter method relies on a multiplex PCR amplification step and is vulnerable to certain issues (allele dropout, incorrect assessment of variant allele frequencies (VAF), potential miscalling of variants in poor quality regions, inconsistent coverage of different regions, …) (11).

The targeted enrichment method used in our laboratory is termed "Anchored Multiplex PCR (AMP)" and is considered as a mixture of the two previously described approaches. Briefly, fragmented DNA is ligated with Unique Molecular Index (UMI) adaptors to label each DNA molecule with a unique number, followed by PCR amplification with custom designed gene specific primers and one universal primer complementary to the present adaptor. Finally, sample index primers are used to create a sequencing-ready library. This library pool is sequenced on an Illumina MiSeq micro or standard V2 flow cell (300 cycli) (12). A brief overview of these three techniques is shown in Figure 1.

The AMP assay is nowadays used in routine in a NGS panel to investigate myeloid neoplasms (21 and 141 gene panel) and to investigate TP53 and IDH2 mutations. It is estimated that this new lymphoid gene panel will contain around 50 genes, making this current technique suitable.

Figure 1. Comparison of amplicon-based, anchored multiplex PCR (AMP)-based and hybridization capture-based NGS techniques



1.2 Composition lymphoid panel

With this information in mind, a thorough literature search of published data of recurrently mutated genes regarding somatic mutations in mature lymphoid malignancies in humans is conducted. Studies who proposed or tested a gene panel themselves are included, commercially available panels or panels used in other clinical laboratories are consulted too. Afterwards, the size of the selected exons of interest co-determine if a gene could be included. Finally, input from the clinical haematologists in our centre is gathered to obtain a final gene panel.

Two main publications have delivered an essential contribution to the composition of our panel. The first one is a review article by ROSENQUIST et al. (Haematologica, 2016) (8), in which the European Expert Group on NGS-based Diagnostics in Lymphomas (EGNL) subdivide about 30 gene mutations in 'immediate impact on treatment decisions', 'diagnostic potential', 'prognostic potential', 'potential clinical impact in the near future' and 'research purposes only'. The second import study is from the French LYSA (LYmphoma Study Association) and GBMHM (Groupe de Biologistes Moléculaires des Hémopathies Malignes) (SUJOBERT et al., HemaSphere 2019) (1). Two consensus panels for B - and T lineage lymphomas are identified consisting of 33 respectively 11 genes, based on clinical relevant mutations or copy number variations. Data on exons to be sequenced is available too.

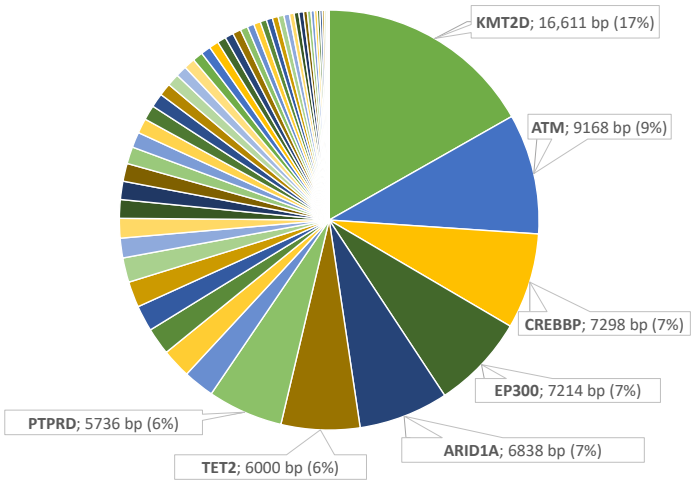
For B-cell lymphomas, two other studies which developed a gene panel for DLBCL (9) and for CLL, FL and DLBCL (3) are included. Besides, one meta-study regarding CLL (6), two commercially available panels (13,14) and one panel used in another clinical laboratory (15) are included. Altogether, 95 different genes mutation are used as starting number. All these are checked for clinical relevance in various review studies (2,5,10,16–20).

As shown in attachment 1, 21 genes are excluded which occurred in only one gene panel without any further reference. Another 20 genes are excluded of which no clear diagnostic, prognostic or therapeutic information could be retained in the various review studies. At this point, the panel includes 54 genes mutation with substantial evidence of diagnostic, prognostic or therapeutic value.

Next, all exons of interest are determined based on supplemental information of several publications (1,3,9,15). For some genes, additional sources are consulted since a discrepancy of which exons should be sequenced was noticed between the various publications. All genes are finally consulted in the "Catalogue of Somatic Mutation in Cancer" (COSMIC) database (available via https://cancer.sanger.ac.uk/cosmic) to confirm the selection of exons.

As such, the previously mentioned 54 genes results in 98,933 base pairs. As shown in Figure 2, 59% of all base pairs are occupied by only seven different genes (KMT2D, ATM, CREBBP, EP300, ARID1A, TET2, PTPRD). For technical and financial reasons, these genes were re-evaluated for clinical significance.

Figure 2. Pie chart of total base pairs (bp) of all exons of interest (98,993) of 54 selected genes



The decision is made to exclude KMT2D, although frequently reported in FL (~78%), DLBCL (~22-40%) and MCL (~12-23%), there is no sufficient clinical evidence to include this large gene (16,611 bp to be sequenced), besides a potential drug target in the future (1–3,9). The gene ATM is removed too, although commonly reported in CLL (~6-15%, adverse prognosis), MCL (~40-50%, potential therapeutic interest) and SMZL (~6%), there is still insufficient evidence to use mutations in this large gene (9,168 bp to be sequenced) as prognostic marker (1–3,6,8). Finally, PTPRD is excluded in the panel, since only one study mentions mutations in this gene (~20% of SMZL, 5,736 bp to be sequenced), but these mutations were not retrieved in other studies (21,22). In contrast, ARID1A, CREBBP, EP300 and TET2 are preserved in the final panel.

The exclusion of these three genes results in a panel gene panel of 51 genes, consisting of 67,418 base pairs. An overview of selected exons and number of selected base pairs per gene is shown in Figure 3 and Figure 4. A summary of all clinical data of these 51 genes is presented in attachment 2. In this summary, a gradation score is used to reflect clinical relevance for each gene for each lymphoma entity. This score system is based on the publication by SUJOBERT et al (1). For the diagnostic interest of a gene, a score of zero is assigned if the finding of a mutation in that gene has no additional diagnostic value for that lymphoma entity, a score of one if there is a partial diagnostic value and a score of two if it concerns a clear pathognomic mutation. For the prognostic interest, a score of zero is assigned if there is currently no evidence of any prognostic importance of that mutation, a score of one if there is a potential prognostic importance and a score of two if a prognostic importance is confirmed by two independent studies. Thirdly, for the therapeutic interest of gene, a score of zero is assigned if there are no therapeutic implications of that mutation at this moment, a score of one if there is a potential therapeutic interest and a score of two if a mutation can be used as predictive biomarker. Finally, this gene panel is proposed to the five clinical haematologists in our centre. No necessary additions are noted.

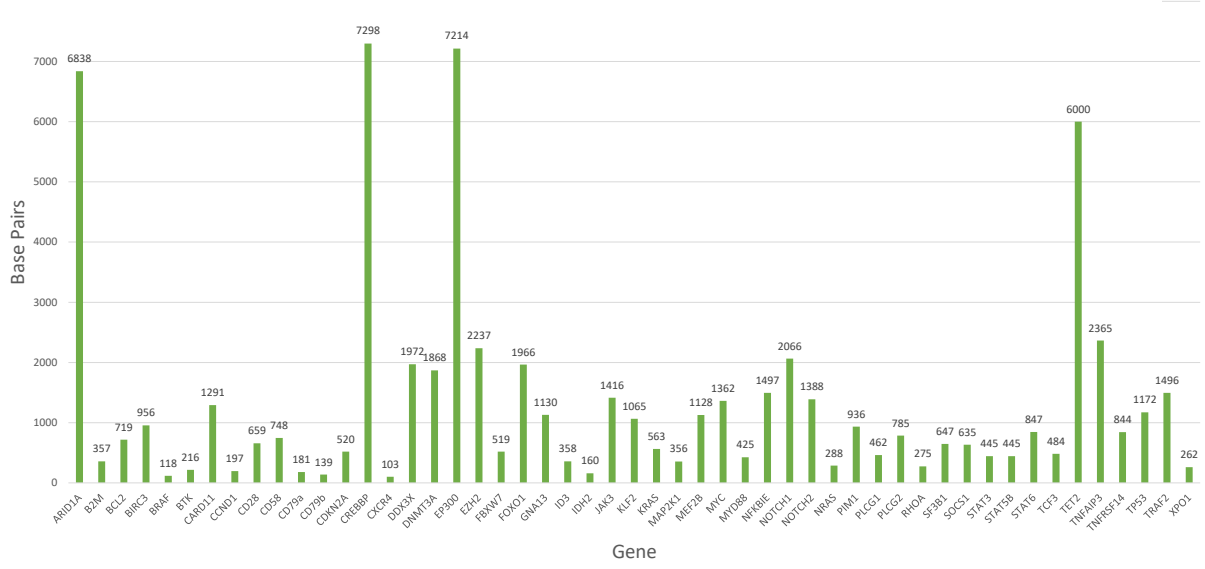
Next, the "Genome Reference Consortium Human genome build 37" (GRCH37/hg19) is consulted (available via http://grch37.ensembl.org/index.html) to determine the genomic coordinates of all coding exons of interest, including transcript ID and RefSeq. This information is provided to the QIAGEN Bioinformatics Support Department to design the necessary QIAseq primers. Their design consists of 928 primers and is subsequently verified via the Integrative Genomics Viewer (IGV) (available via http://software.broadinstitute.org/software/igv/) to visualize the primers on the reference genome and compare them with RefSeq transcripts. As such, artefacts and sequencing errors can be excluded. Finally, the lymphoid 51-gene panel is ready to be ordered created as a QIASeq Targeted DNA custom panel.

Figure 3. Overview of final selected genes including exons of interest, transcript number and refseq (GRCH37/hg19)

Gene	Exon	transcript	refseq
ARID1A	1-20	ENST00000324856.7	NM_006015
B2M	1-3	ENST00000558401.1	NM_004048
BCL2	1-2	ENST00000398117.1	NM_000633
BIRC3	4-10	ENST00000532808.1	NM_182962
BRAF	15	ENST00000288602.6	NM_004333
BTK	15	ENST00000308731.7	NM_000061
CARD11	4-10	ENST00000396946.4	NM_032415
CCND1	1	ENST00000227507.2	NM_053056
CD28	1-4	ENST00000324106.8	NM_001243077
CD58	1-6	ENST00000369489.5	NM_001779
CD79a	4-5	ENST00000221972.3	NM_001783
CD79b	5-6	ENST00000392795.3	NM_001039933
CDKN2A	1-2	ENST00000361570.3	NM_058195
CREBBP	1-31	ENST00000262367.5	NM_004380
CXCR4	2	ENST00000241393.3	NM_003467
DDX3X	1-17	ENST00000399959.2	NM_001193416
DNMT3A	8-23	ENST00000264709.3	NM_17562
EP300	1-31	ENST00000263253.7	NM_001429
EZH2	2-20	ENST00000320356.2	NM_001203247
FBXW7	8-10	ENST00000281708.4	NM_033632
FOXO1	1-2	ENST00000379561.5	NM_002015
GNA13	1-4	ENST00000439174.2	NM_006572
ID3	1-2	ENST00000374561.5	NM_002167
IDH2	4	ENST00000330062.3	NM_002168
JAK3	10-19	ENST00000458235.1	NM_000215
KLF2	1-3	ENST00000248071.5	NM_016270

Gene	Exon	transcript	refseq
KRAS	2-5	ENST00000311936.3	NM_004985
MAP2K1	2-3	ENST00000307102.5	NM_002755
MEF2B	2-9	ENST00000424583.2	NM_001145785
MYC	1-3	ENST00000377970.2	NM_002467
MYD88	3-5	ENST00000396334.3	NM_002468
NFKBIE	1-6	ENST00000275015.5	NM_004556
NOTCH1	26-27,34	ENST00000277541.6	NM_017617
NOTCH2	34	ENST00000256646.2	NM_024408
NRAS	2-3	ENST00000369535.4	NM_002524
PIM1	1-6	ENST00000373509.5	NM_001243186
PLCG1	1,11,29	ENST00000373272.2	NM_002660
PLCG2	19,20,22,24,26,27	ENST00000359376.3	NM_002661
RHOA	2-3	ENST00000418115.1	NM_001664
SF3B1	13-16	ENST00000335508.6	NM_012433
SOCS1	2	ENST00000332029.2	NM_003745
STAT3	19-21	ENST00000264657.5	NM_139276
STAT5B	14-17	ENST00000293328.3	NM_012448
STAT6	12-18	ENST00000300134.3	NM_001178078
TCF3	16-18	ENST00000344749.5	NM_001136139
TET2	3-11	ENST00000380013.4	NM_001127208
TNFAIP3	2-9	ENST00000237289.4	NM_001270507
TNFRSF14	1-8	ENST00000355716.4	NM_003820
TP53	2-11	ENST00000269305.4	NM_000546
TRAF2	2-11	ENST00000247668.2	NM_021138
XPO1	15,19	ENST00000401558.2	NM_003400

Figure 4. Overview of final selected genes with total base pairs to be sequenced



***Question 2.* How can such panel be implemented in routine clinical practice?**

With the knowledge of the purpose of this new assay, acceptable clinical sample types and rationale for the inclusion of specific genes, it is of interest which methodological approach will be used to validate this panel (11). A brief overview of the verification procedure will be highlighted, accompanied by a methodology of variant interpretation. Furthermore, total costs and reimbursement will be calculated and discussed. Finally, a strategy will be worked out to determine which patients will be tested with this new assay.

2.1 Verification plan

The new lymphoid panel will use a similar method as the ISO15189 accredited QIASeq custom myeloid 21 gene panel, including library preparation, sequencing and data-analysis. The only change will be an adapted QIASeq targeted primer panel during target enrichment and an adaption of the regions of interest (ROI) during data-analysis. Therefore, performing a verification study will be sufficient.

Acceptable performance conditions are based on BELAC document 2-405 (23) and are shown in Table 1. An artificial sample will be created consisting of a dilution of different samples, with finally 10 known mutations present at a target VAF value of ~5%. The known mutations will be in genes BRAF, CXCR4, MYD88, NOTCH1, STAT3 and TP53, since these are previously determined with NGS, Sanger sequencing or another PCR technique. This artificial sample will be used to conduct the precision (reproducibility) - and accuracy study. An interference study will be conducted too by combing the libraries of the QIASeq Custom Myeloid panel and Lymphoid panel in one flow cell.

Besides this artificial sample, at least ten patient samples will be retrospectively tested with known diagnosis and therapeutic outcome. These samples will include at least two peripheral blood, bone marrow and lymph node samples to determine if extracted DNA from these different sample types is of equally good quality. This information will be helpful to establish experience with interpretation of the variants.

Table 1. Acceptable performance conditions

	Acceptance criteria
Precision	- Variant with a VAF ≥5% always detectable - VAF max 10% different
Accuracy	- EQC samples: VAF ≥5% always detectable - EQC samples: VAF max 10% different - Variants detected by Sanger sequencing always detectable - No false positives
Measuring range & linearity	- Rico and intercept not significantly different from one respectively zero
Limit of Detection	- 5% mutant DNA: detected in 95% of all cases

2.2 Variant analysis

In 2019, the Belgium next-generation sequencing guidelines for haematological and solid tumours are published to facilitate and harmonise implementation, verification and validation of targeted NGS tests (23). Since no clear guidelines concerning biological classification of somatic mutation were available, the Belgian 'Commission of Personalized Medicine' (ComPerMed) set up an expert group to introduce Belgian guidelines of this topic (24). The result is a *biological* variant classification workflow, based on the five biological classes of the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) Standards and Guidelines, and a *clinical* classification scoring system, according to the four-tiered ACMG/AMP guideline system. The expert group has mainly focused on NGS variants of solid and haematological tumours. Briefly, the biological variant classification workflow, which is shown in attachment 3, is a five step process wherein each detected variant will be classified as benign, likely benign, variant of unknown significance (VUS), likely pathogenic or pathogenic.

Although this workflow is designed so it can be used independent of panel design, three remarks can be made when this workflow is applied specifically for mature lymphoid malignancies. First, step three of the workflow (see attachment 3) is to check if a somatic variant is included in the 'Consensus Pathogenic Variants' (CPV) list. This list includes only the ComPerMed genes selected for screening in solid and in myeloid tumours. Somatic mutations in the lymphoid panel will be less classified as 'pathogenic', since variants in genes not present in the CPV list need to obtain a +3.5 score in the Scoring Table (step five of the workflow) to classify them as 'pathogenic'. So, before this workflow can be used, it is a requirement that the CPV list is extended for mature lymphoid tumours. A temporary list can be established in our laboratory as long as no extension is made.

Secondly, as mentioned above, the fifth step of the workflow includes a scoring system based on four parameters (see attachment 3) to classify mutations in VUS (score <2) or Likely Pathogenic (score ≥2). The parameter with the most substantial contribution is "the total entries of that particular amino acid change at that position in COSMIC". The selected ComPerMed genes for myeloid tumours are long-established, well-studied genes. This ensures total entries in COSMIC are relatively high. The selected genes in our new panel are often less 'popular' which means detected variants will have much less, sometimes no hits in COSMIC. One could argue that an adaption of this 'total entries' in this score table is needed for mature lymphoid malignancies. Additionally, concerning the remaining parameters of the score system, it should be noted that no specific database is available for lymphoid malignancies and the extension of existing ones is needed (25). A final remark regarding the workflow system concerns the fourth step, whereby a clear loss-of-function (LoF) mutation in a oncogene is classified as VUS versus in a tumour suppressor gene as Likely Pathogenic. This list of oncogenes and tumour suppressor genes lacks several genes who are included in our lymphoid panel, indicating this list is not fully completed for lymphomas and needs to be extended.

With this information in mind, an update of this guidelines is needed for mature lymphoid malignancies and will be useful if more laboratories will introduce a mature lymphoid NGS panel.

2.3 Costs & Reimbursement

With the introduction of the myeloid 21 gene panel, a calculation of the total costs per sample was conducted. Since no changes will be made in the workflow process with this new lymphoid panel compared to the myeloid panel, the only changing cost is the price of the QIASeq Target DNA custom panel and the higher sequencing cost. A summary of the total costs per sample is shown in Table 2. Of interest: the costs for instruments, bioinformatics software and maintenance contracts is calculated on 500 samples (different gene panels) per year, the working time is calculated per batch of 10 samples, the validation study based on 80 samples and the external - and internal quality control on 10 samples per year each.

This calculation yields a price of €361 per sample. However, including Belgian value-added tax rate of 21% and overhead costs (56% on indirect cost, based on general hospital interventions (26)), yields a price of €681 per sample. To compare: the total cost per sample using the myeloid panel is €240 (without VAT and overhead costs) and €452 including VAT and overhead costs.

Table 2. Calculation of price per sample analysed by the lymphoid NGS panel

	Price per sample (€)
Extraction	3.63
Concentration calculation	0.66
QIASeq (custom panel + index set)	82.24
Sequencing	127.87
Instruments	18.89
Bioinformatic software	8.84
Maintenance contracts	10.87
Working time	64.68
validation & quality control	42.96
Total	**360.65**
21% VAT + 56,6% overhead costs	680.76

To calculate the reimbursement of each NGS analysis for the laboratory, the fee of article 33 bis (tests with diagnostic, prognostic and therapeutic interest without link to a specific medicine) and 33 ter (tests linked to a specific medicine) concerning genetic studies are needed. Neither mature B cell lymphoma nor T cell lymphoma are eligible for an additional surcharge from the NGS convention at this time, unlike for many myeloid malignancies (surcharge of €350 can be added). A summary of the fees that can be charged for this new lymphoid panel is shown in Table 3 (B-cell lymphomas) and Table 4 (T-cell lymphomas). Herein, the following medical provisions according to article 33 bis are included: 588453-588464 and 588475-588486. In case of CLL, the following medical provision according to article 33 ter are included: 594053-594064 and 594090-594101. The table is established as follow: in case of FL for example, molecular analysis of IGH and IGK are conducted and are charged via provision rules 558475-588486 (article 33 bis). Besides, t(14;18)/BCL2 is analysed by FISH and BCL2 mutation by PCR (three qualitative multiplex PCR reactions). According to provisions 588453-588464 of article 33 bis, these provisions can be charged three times per diagnostic investigation, so there's one remaining rule of art. 33 bis left if the lymphoid NGS panel will be tested.

Table 3. Overview of medical provisions selected from article 33 bis and 33 ter for mature B-cell lymphomas

	Provisions art. 33 bis	Max. rules art. 33 bis	Max. rules art. 33 ter	Current molecular assay	Current technique	Replaceable tests by lymphoid NGS panel	Extra reimbursement in respect to current reimbursement
B-NHL - general	588475-588486	2	0	IGH	PCR	N.A.	
				IGK			
CLL	588453-588464	3	2 *	TP53 / del(17)(p13)	FISH	TP53	3 x art. 33 bis
				TP53	NGS		
MCL	588453-588464	3	0	TP53 / del(17)(p13)	FISH	TP53	0
				BCL1-IgH / t(11;14)			
				TP53	NGS		
DLBCL	588453-588464	3	0	cMYC / t(8q24)	FISH	0	0
				BCL2-IgH / t(14;18)			
				BCL6 / t(3q27)			
				BCL2	PCR		
Burkitt	588453-588464	5	0	cMYC / t(8q24)	FISH	0	2 x art. 33 bis
				BCL2-IgH / t(14;18)			
				BCL6 / t(3q27)			
FL	588453-588464	3	0	BCL2-IgH / t(14;18)	FISH	0	1 x art. 33 bis
				BCL2	PCR		
HCL	588453-588464	3	0	BRAF	PCR	BRAF	2 x art. 33 bis
LPL	588453-588464	3	0	MYD88	PCR	MYD88	2 x art. 33 bis
SLVL	588453-588464	3	0	del(7q)(q22-q36)	FISH	0	2 x art. 33 bis
MALT	588453-588464	3	0	MALT1 / t(18q21)	FISH	0	2 x art. 33 bis
B-NHL, other	588453-588464	3	0	0	0	0	3 x art. 33 bis

* provision rules 594053-594064 and 594090-594101

Table 4. Overview of medical provisions selected from article 33 bis and 33 ter for mature T-cell lymphomas

	Provisions art. 33 bis	Max rules art. 33 bis	Max rules art. 33 ter	Current molecular assay	Current technique	Replaceable tests by lymphoid NGS panel	Extra reimbursement in respect to current reimbursement
T-NHL - general	588475-588486	2	0	TCR	PCR	N.A.	
ALCL *	588453-588464	3	0	0	0	0	3 x art. 33 bis
T-LGL	588453-588464	3	0	STAT3	Sanger	STAT3	2 x art. 33 bis
ALTCL	588453-588464	3	0	t(2p23)/ALK	FISH	0	2 x art. 33 bis
T-NHL, other	588453-588464	3	0	0	0	0	3 x art. 33 bis
* provision rules 588475-588486 are applicable too							

As such, the partial reimbursement to the laboratory is different for each lymphoma entity. General, three situations may occur. First, in case of DLBCL for example, the full cost of analysing the panel will be covered by the laboratory. Secondly, in case of ALCL for example, provisions 588453-588464 can be charged three times when analysing the panel. Thirdly, in case of T-LGL for example, STAT3 is now analysed by Sanger sequencing. This can be replaced by the new NGS panel, so the Sanger assay can be saved. Provision 588453-588464 can be charged additionally two times when analysing the panel in respect to current reimbursement. However, together with the total costs of the lymphoid panel as described above, the laboratory will always have to make a financial commitment when performing the new panel.

2.4 Clinical indications

In consultation with the clinical haematologists, a workflow is designed which patients will be tested in routine clinical practice. In the first place, the panel will be used in patients whereby the tentative diagnosis is still inconclusive with the current diagnostic tools or when discrepancies occur between the various techniques. Alternatively, lymphomas of very rare entities will also be tested from a scientific point of view (and/or confirmatory diagnostic reasons). By definition, the latter will only be a very small number of cases. The decision to perform the analysis will be made during the multidisciplinary oncologic consult (MOC), in which physicians form different medical subdisciplines participate (clinical haematology, pathology, nuclear medicine, clinical biology). The age of the patient will not be taken into account as there is no specific financial compensation from the NGS convention for these indications. It is expected that this will be more often the case in mature T-cell lymphomas, since clonality with the currently used T-Cell Receptor Gene Rearrangement is unclear from time to time. The turn-around time will be the same as the NGS myeloid panel, *i.e.* maximum 28 days.

One could argue that the use of this panel is an overshooting for such indications, since the panel includes genes for both B – and T-cell lymphomas. In addition, the panel includes various genes with prognostic or therapeutic implication who are not yet included in the recent guidelines and are as such not yet applied in the current choice of therapy. However, the validation costs of such panel are relative high, so no new panel can be designed every time new guidelines appear. In this point of view, it is expected that the clinical indications for which the panel is used will be expanded as soon as new guidelines or new targeted therapies (possibly in the context of a clinical study) are available for the various mature B – and T-cell lymphomas.

Conclusion

Based on extensive scientific literature, a customized QIASeq targeted DNA custom panel of 51 genes with diagnostic, prognostic and therapeutic interest of mature lymphoid malignancies is composed. The panel consist of 67,418 base pairs which are covered by 928 primers and will be analysed by Anchord Multiplex PCR (AMP) next-generation sequencing on a MiSeq System. Patients bone marrow specimen or fresh tissue material will be tested with this panel in case of an inconclusive diagnosis with current diagnostic tools. This decision will be made during multidisciplinary oncologic consult. It is expected that the variants gathered with the panel can aid the clinician in his/her patient management. By deciding during MOC consultation which patient will be tested, an overuse of the panel is attempted to be avoided.

To do/Actions

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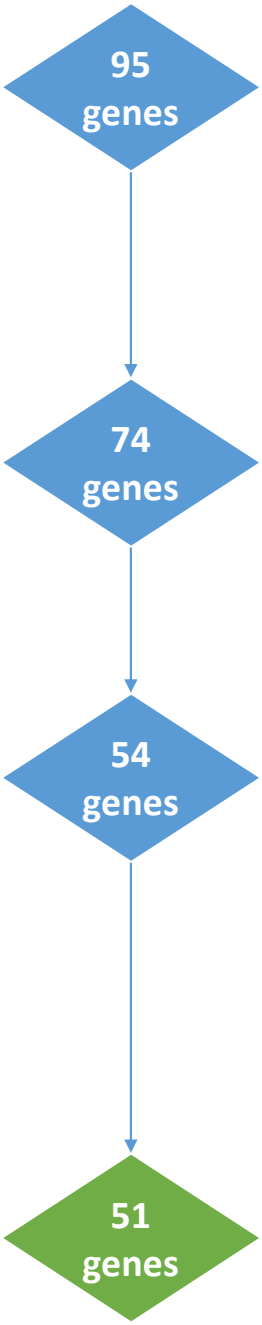
1) Performance of the verification study

2) A side project of this new method in the diagnosis and follow-up of mature lymphoid malignancies is the composition of a second panel, which will use cell free DNA to investigate lymphomas in a patients peripheral blood. For this second panel, a selection must be made of this lymphoid panel with genes that are most suitable for the detection in cell free DNA.

Attachment 1. Workflow of gene selection



Exclusion of genes based on:
occurence in only one gene panel without any references

BCL6	FGFR3	ITPKB	PRKDC
BCL10	GNAI2	JAK1	SYK
CDH2	IDH1	JAK2	TMEM30A
FAS	IKZF1	KMT2C	
FGFR1	IKZF3	MTOR	
FGFR2	IRF8	PDGFRA	

Exclusion of genes based on:
No clear diagnostic/prognostic/theranostic information

BTG1	KIT	POT1	SGK1
CCND3	KLHL6	PRDM1/BLIMP1	ZMYM3
CDKN2B	MAPK7	PTEN	
CHD2	MFHAS1	PTPN1	
CIITA	PIK3CA	PTPN6	
IRF4/MUM1	PIK3CD	SAMHD1	

Exclusion of genes based on:
Size of the gene does not outweigh the diagnostic/prognostic/theranostic information

ATM[2]	KMT2D/MLL2	PTPRD[1]

Genes included

ARID1A[1]	CXCR4[1,2]	MEF2B[1]	STAT5B[1]
B2M[1]	DDX3X[2]	MYC[1]	STAT6[1]
BCL2[1]	DNMT3A[1,2]	MYD88[1,2]	TCF3[1,2]
BIRC3[1,2]	EP300[1]	NFKBIE[1,2]	TET2[1,2]
BRAF[1,2]	EZH2[1,2]	NOTCH1[1,2]	TNFAIP3[1]
BTK[1,2]	FBXW7	NOTCH2[1,2]	TNFRSF14
CARD11[1,2]	FOXO1[1]	NRAS	TP53[1,2]
CCND1[1]	GNA13	PIM1	TRAF2[1]
CD28[1]	ID3[1,2]	PLCG1[1]	XPO1[1]
CD58	IDH2[1,2]	PLCG2[1,2]	
CD79A[1]	JAK3[1]	RHOA[1,2]	
CD79B[1,2]	KLF2[1,2]	SF3B1[1,2]	
CDKN2A[1]	KRAS	SOCS1	
CREBBP[1]	MAP2K1	STAT3[1,2]	

[1] included in panel of SUJOBERT et al.
[2] Included in panel of ROSENQUIST et al.

Attachment 2. Summary of clinical information of selected genes (in dutch)

Toelichting klinische gegevens NGS 51 genen Lymfoïd Panel

Klinische revelantie score

	0	1	2
diagnostisch belang	geen	deels	duidelijk (pathognomonische mutatie)
prognostisch belang	geen	mogelijks	bevestigd (in ten minste 2 studies)
theranostisch belang	geen	mogelijks	bevestigd (predictieve biomerker)

Afkortingen

ABC = activated B cell
AITL = angio-immunoblastic T lymphoma
ALCL = anaplastic large cell lymphoma
ATLL = adult T leukemia/lymphoma
BL = Burkitt lymphoma
CLL = chronic lymphocytic leukemia
CNV = copy number variation
DLBCL = diffuse large B cell lymphoma
EATL = enteropathy associated T lymphoma
FL = follicular lymphoma
tFL= transformed folliculair lymfoom
GC = germinal center
HCL = hairy cell lymphoma
HL = Hodgkin lymphoma
HSTL = hepatosplenic T lymphoma
LGL = large granular lymphocytic leukemia
MCL = mantle cell lymphoma
MEITL = monomorphic epitheliotropic intestinal T lymphoma
MZL = marginal zone lymphoma
NKTCL = nasal type NK/T cell lymphoma
OC = oncogene
PCNSL = primary central nervous system lymphoma
PMBCL = primary mediastinal B cell lymphoma
PTCL-NOS = peripheral T cell lymphoma, not otherwise specified
PTCL-TFH = nodal peripheral T cell lymphoma derived from TFH cells
Sezary = Sezary syndrome
T-PLL = T-prolymphocytic leukemia
TSG = Tumour Suppressor Gene
WM = Waldenström macroglobulinemia

Functie gen	Gen	Type	pathologie	mutatie frequentie (%)	CNV frequentie (%)	Diagnostisch belang	Prognostisch belang	Therapeutisch belang	Referentie
Epigenetische modicifatie									
	ARID1A	TSG	CLL	1		0	0	1	Sujobert 2018, Hung 2018, Rosenquist 2017
			FL	11-15		0	2 (M7-FLIPI)	1	
			tFL	15					Rosenquist 2017
			SMZL	5		0	0	1	Sujobert 2018
			WM	17		0	0	1	
			DLBCL	4-8		?	?	?	Hung 2018, Rosenquist 2017
			NKTCL	5		?	?	?	Zhang 2018
			HCL	5-10		1	?	?	
	CREBBP	TSG	FL	60		0	2 (M7-FLIPI)	1	Sujobert 2018, Dubois 2016, Hung 2018, Scott 2018
			DLBCL-ABC	13		0	0	0	
			DLBCL-GCB	15		0	0	0	
			CLL	1		0	0	0	Sujobert 2018
			BL	12		0	0	0	
	DNMT3A	TSG	AITL/PTCL	8-30		1	?	?	Sujobert 2018, Rosenquist 2016
			PTCL NOS	15		?	?	?	
			HSTL	8		?	?	?	
			Sezary	15		?	?	?	
			ATLL	2		?	?	?	
	EP300	TSG	DLBCL-ABC	4		1	0	0	Sujobert 2018, Scott 2018, Dubois 2016, Hung 2018, Rosenquist 2017
			DLBCL-GCB	1		1	0	0	
			FL	8-19		0	2	1	
			SMZL/SRPL	5		0	0	0	
			NKTCL	0-5		1	?	?	Zhang 2018
	EZH2	TSG / OC	DLBCL-ABC	0-4		1	0	1	Sujobert 2018, Rosenquist 2016, Hung 2018
			DLBCL-GCB	5-25		1	0	1	
			FL	7-27		0	2 (m7-FLIPI)	1 (toekomst: EZH2 inhibitor)	
	IDH2	OC	AITL	30		1	0	1 (toekomst: IDH2 inhibitoren)	Sujobert 2018, Rosenquist 2016
			HSTL	5		?	?	?	
			ATLL	1		?	?	?	
	TET2	TSG	AITL/PTCL-TFH	50-65		2	2 (agressievere presentatie, korte progression-free survival)	0	Sujobert 2018, Rosenquist 2016
			ATLL	10		?	?	?	
			HSTL	8		?	?	?	
			PTCL NOS	17-25		?	?	?	
			T-PLL	17		?	?	?	
			EATL	15		?	?	?	
			Sezary	15		?	?	?	
Immuniteit ontwijking									
	B2M	TSG	DLBCL	10-23		?	2 (meer relapses)	?	Hung 2018, Rosenquist 2017
			DLBCL-ABC	5-9		0	1	0	Dubois 2016, Sujobert 2018
			DLBCL-GCB	5-18		0	1	0	
			PMBL	18		0	0	0	
			HL	70		0	2	0	Sujobert 2018
	CD58	TSG	DLBCL (PMBL)	5		?	?	1 (mutaties mogelijk effect op PD-1 inhibitor)	Dubois 2016, Scott 2018
			FL	5		?	?	?	Bogusz 2016
	TNFRSF14	TSG	DLBCL	14		?	1 (slechte prognose)	?	Dubois 2016, Hung 2018, Rosenquist 2017
			FL	30		?	1 (slechte prognose)	?	

Functie gen	Gen	Type	pathologie	mutatie frequentie (%)	CNV frequentie (%)	Diagnostisch belang	Prognostisch belang	Therapeutisch belang	Referentie
Anti-apoptotisch / Cel cyclus regulatie									
	BCL2	OC	FL	50		0	1	1	Sujobert 2018
			tFL	47					Rosenquist 2017
			DLBCL-ABC	1		0	0	1	Sujobert 2018, Dubois 2016
			DLBCL-GCB	10-24		0	0	1	Sujobert 2018, Dubois 2016
			Opmerking: t(14;18) zorgt voor overexpressie, waardoor maligne cellen langer kunnen overleven (i.e. initieel "oncogenic hit")						Scott 2018, Rosenquist 2017
	CCND1	OC	MCL	14-34	9	1	1	2 (resistentie aan ibrutinib)	Sujobert 2018, Rosenquist 2017
			Opmerking: 95% van MCL heeft translocatie van CCND1/BCL1(11q13) naar IgH gen locus (14q32) waardoor CCND1-overexpressie ontstaat						Rosenquist 2017
	CDKN2A	TSG	MCL		25	0	2 (slechte prognose)	1 (slechte behandeling met standaard chemo, toekomst PRMT5 inhibitoren?)	Sujobert 2018, Rosenquist 2017
			PMBL		9	1	0	0	Sujobert 2018
			DLBCL(-ABC)		35	0	2 (slechte prognose)	0	Sujobert 2018, (Dubois 2016), Iqbal et al 2016
			PCNSL		45-80	0	0	0	Sujobert 2018
	MYC	OC	MCL		18	0	1 (slechte prognose)		Sujobert 2018, Dubois 2016
			BL	41		0	0	0	
			DLBCL-ABC	1		1	0	0	
			DLBCL-GCB	6		1	0	0	
			PMBL	25-31		0	0	0	
	TP53	TSG	BL	20-25		0	0	1	Sujobert 2018, Rosenquist 2016, Hung 2018, Dubois 2016, Bogusz 2016
			CLL	5-10		0	2 (slechte outcome, agressief, refractair aan chemo)	2	
			DLBCL-ABC	9		0	1	1	
			DLBCL-GCB	5		0	1	1	
			FL	7-11		0	1	0	
			HCL	26		0	0	0	
			HCL variant	20		0	0	1	
			HL	12		0	0	0	
			MCL	11-24		0	1	1 (tot 25% in blastaire variant; slechte prognose)	
			MZL	22		0	0	0	
			PMBL	13		0	0	0	
			SMZL	15		0	1	1	
			WM	7		0	0	0	
			EATL	12		?	?	?	Sujobert 2018
			MEITL	15-30		?	?	?	
			Sezary	24		?	?	?	
			ATLL	15		?	?	?	
			T-PLL	14		?	?	?	
			HSTL	10		?	?	?	
			PTCL NOS	8		?	?	?	
			NKTCL	17		?	1 (vergevorderd stadium, slechte prognose)	?	Zhang 2018
Transcriptie factoren									
	FOXO1	TSG	DLBCL	8		0	2 (verminderde overall survival in patiënten behandeld met R-CHOP)	0	Sujobert 2018, Dubois 2016, Hung 2018, Iqbal 2016
			FL	7		0	2 (m7-FLIPI)	0	
	ID3	TSG	BL	68		2	0	0	Sujobert 2018, Rosenquist 2016, Hung 2018
			nodal MZL	9		0	0	0	

Functie gen	Gen	Type	pathologie	mutatie frequentie (%)	CNV frequentie (%)	Diagnostisch belang	Prognostisch belang	Therapeutisch belang	Referentie
Transcriptie factoren									
	KLF2	TSG	DLBCL-ABC	5		1	0	0	Sujobert 2018, Rosenquist 2016
			nodal MZL	17		1	0	0	
			FL	9		0	0	0	
			HCL	10		0	0	0	
			MCL	9		0	0	0	
			SMZL	21-42		2	0	0	
	MEF2B	OC	DLBCL-ABC	5		0	0	0	Sujobert 2018, Dubois 2016, Hung 2018, Rosenquist 2017
			DLBCL-GCB	12		0	0	0	
			FL	7-18		0	2 (m7 FLIPI)	0	
			MCL	3-7		0	0	0	
			PTL	33		0	0	0	
	TCF3	OC	BL	11		2	0	0	Sujobert 2018, Rosenquist 2016, Hung 2018, Dubois 2016
Signalisatie pathways									
NFkB	**BIRC3**	TSG	MCL	3-10		0	1	2 (mogelijks resistent aan ibrutinib)	Sujobert 2018, Bogusz 2016, Rosenquist 2016, Hung 2018
			CLL	4-9		0	2 (slechte outcome, agressief verloop, refractair aan fludarabine)	1	
			SMZL	5		0	0	2	
MAPK	**BRAF**	OC	HCL	>90		1	0	1	Sujobert 2018
			CLL	2-4		0	0	1	Sujobert 2018
			DLBCL	1		?	?	?	Dubois 2016
BCR	**BTK**	OC	algemeen			0	0	2 (relaps CLL en/of refractair aan ibrutinib)	Sujobert 2018, Rosenquist 2016
NFkB	**CARD11**	OC	FL	11		0	2 (m7-FLIPI)	1	Sujobert 2018, Rosenquist 2016, Dubois 2016, Hung 2018, Rosenquist 2017
			DLBCL-ABC	6-12		0	0	2 (resistentie BTK inhibitoren)	
			DLBCL-GCB	3-12		0	0	1	
			MCL	3-15	10	0	1	2	
			CLL	1-2					Rosenquist 2017
			SMZL	~ 7					
			NMZL	8					
			AITL	5		?	?	?	Sujobert 2018, Rosenquist 2016
			ATLL	25		?	?	?	
			Sezary	6-15		?	?	?	
andere	**CD28**	OC	AITL	10		1	?	mogelijks target PD-1 inhibitor	Sujobert 2018, Rosenquist 2016
			Sezary	5		?	?	?	
			ATLL	4		?	?	?	
BCR	**CD79a**	OC	DLBCL-ABC	3		0	1	1	Sujobert 2018
			FL	6		0	0	1	
			SMZL	1		0	0	1	
BCR	**CD79b**	OC	DLBCL-ABC	21-23		1	0	1 (meer gevoelig aan ibrunitib)	Sujobert 2018, Rosenquist 2016, Hung 2018, Scott 2018
			DLBCL-GCB	1-3		1	0	1	
			PCNSL	64		0	0	1	
			PTL	83		0	0	1	
			WM	7		0	0	1	

Functie gen	Gen	Type	pathologie	mutatie frequentie (%)	CNV frequentie (%)	Diagnostisch belang	Prognostisch belang	Therapeutisch belang	Referentie
Signalisatie pathways									
andere	**CXCR4**	OC	WM	27		1	0	2 (respons ibrutinib afh van mutatie MYD88 en CXCR4)	Sujobert 2018, Rosenquist 2016
			DLBCL	4		?	?	?	Rosenquist 2017
andere	**GNA13**	TSG	DLBCL	11		0	1 (DLBCL-ABC, R/ R-CHOP geeft slechtere progression free survival)	0	Dubois 2016, Hung 2018, Rosenquist 2017
			FL	3		0	0	0	
JAK/STAT	**JAK3**	OC	ATLL	2-11		?	?	1 (JAK3 inhibitoren)	Sujobert 2018, Rosenquist 2016, Zhang 2018
			CTCL/Sezary	3		?	?	?	
			T-PLL	30-40		?	?	?	
			AITL	2		?	?	?	
			EATL	25		?	?	?	
			PTCL NOS	3,8		?	?	?	
			NKT	7-35		?	?	?	
MAPK	**KRAS**	OC	CLL	?		?	?	1 (slechte respons chemo)	Vendrami 2019, Rodriguez-Vicente 2017
MAPK	**MAP2K1**	OC	HCL variant	50		1	0	0	Waterfall 2014, Maitre 2018
TLR	**MYD88**	OC	DLBCL-ABC	13 - 30		2	0	1	Sujobert 2018, Rosenquist 2016, Hung 2018, Dubois 2016, Iqbal 2016, Bogusz 2016
			DLBCL-GC	3		2	0	1	
			PCNSL	38-86		1	0	0	
			PTL	67		0	0	0	
			SMZL	5-21		0	0	0	
			WM	79-91		2	2	2 (cfr. CXCR4; mutatie belangrijk voor respons ibrutinib)	
			CLL	3-4		0	1 (gunstige outcome)	0	
NFkB	**NFKBIE**	TSG	CLL	1		0	1	0	Sujobert 2018, Rosenquist 2016
			HL	27		0	0	0	
			PMBL	23		0	1 (refractair, slechte outcome)	0	
NOTCH	**NOTCH1**	TSG/OC	CLL	8-17		0	1 (slechte outcome, aggressief verloop, refractair aan chemo + hoger risico op Richterse)	1	Sujobert 2018, Rosenquist 2016, Hung 2018, Dubois 2016, Bogusz 2016
			DLBCL-ABC	1		0	0	1	
			DLBCL-GCB	1		0	0	1	
			MCL	5-13		0	2	0	
			SMZL/SRPL	5		0	0	0	
			BL	8		0	0	0	
NOTCH	**NOTCH2**	TSG/OC	CLL	8-17		0	1	1	Sujobert 2018, Rosenquist 2016, Hung 2018, Dubois 2016
			DLBCL-ABC	1		0	0	1	
			DLBCL-GCB	1		0	0	1	
			MCL	5-13		0	2	0	
			SMZL/SRPL	5		0	0	0	
			BL	8		0	0	0	
MAPK	**NRAS**	OC	CLL	?		?	?	1 (slechte respons chemo)	Vendrami 2019, Rodriguez-Vicente 2017
NFkB	**PIM1**	OC	DLBCL (vnl ABC)	18-20		?	?	1 (mogelijk link met PIM kinase inhibitoren)	Dubois 2016, Hung 2018

Functie gen	Gen	Type	pathologie	mutatie frequentie (%)	CNV frequentie (%)	Diagnostisch belang	Prognostisch belang	Therapeutisch belang	Referentie
Signalisatie pathways									
TCR-NF-kB	**PLCG1**	OC	AITL/PTCL	15		?	0	0	Sujobert 2018, Rosenquist 2016
			PTCL-NOS	15		?	0	0	
			ATLL	33		?	0	0	
			CTCL/Sezary	14-21		1	0	0	
trans-membraan	**PLCG2**	OC	algemeen	?		0	0	2 (mutatie wordt gevonden bij ibrutinib resistentie)	Sujobert 2018, Rosenquist 2016
andere	**RHOA**	TSG/OC	AITL/PTCL	34-67		2		0	Sujobert 2018, Rosenquist 2016
			ATLL	12		?	0	0	
			PTCL NOS	15-18		?	0	0	
			Sezary	7		?	0	0	
JAK-STAT	**SOCS1**	TSG	PMBCL	15-50		0	0	1 (ruxolitinib mogelijks effectief)	Dubois 2016, Iqbal 2016
JAK-STAT	**STAT3**	OC	LGL	36-40		2	?	?	Sujobert 2018, Rosenquist 2016, Zhang 2018
			AITL	2		?	?	?	
			ALK neg ALCL	20		?	?	?	
			ATLL	23		?	?	?	
			HSTL	10		?	?	?	
			EATL	20		?	?	?	
			MEITL	10		?	?	?	
			NKTCL	14-25		?	?	1 (onderzoek naar STAT3 inhibitoren)	
			CTCL/Sezary	3-21		?	?	?	
JAK-STAT	**STAT5B**	OC	EATL	10		2	?	?	Sujobert 2018, Rosenquist 2016
			MEITL	30-65		?	?	?	
			HSTL	30		?	?	?	
			T-PLL	36		?	?	?	
			NKTCL	6		?	?	?	
JAK-STAT	**STAT6**	OC	PMBL	36		1	1	1 (Ruxolitinib mogelijks effectief)	Sujobert 2018, Dubois 2016, Hung 2018, Iqbal 2016
			FL	12		0	0	0	
NFkB	**TNFAIP3**	TSG	DLBCL-ABC	15-26		1	1 (R-CHOP: slechte prognose)	1 (verminderde activiteit ibrutinib en sotrastaurin)	Sujobert 2018, Dubois 2016
			DLBCL-GCB	2-11		1	1	1 (verminderde activiteit ibrutinib en sotrastaurin)	
			FL	11		0	0	1	
			HL	44-60		0	0	1	
			nodal MZL	15		0	0	0	
			PMBL	36		0	0	0	
			SMZL	7		0	0	1	
NFkB	**TRAF2**	OC	CLL	1		0	0	1	Sujobert 2018
			DLBCL-ABC	3		0	0	1	
			DLBCL-GCB	9		0	0	1	
			MCL	6		0	0	2 (resistentie ibrunitib)	
			WM	3		0	0	1	

Functie gen	Gen	Type	pathologie	mutatie frequentie (%)	CNV frequentie (%)	Diagnostisch belang	Prognostisch belang	Therapeutisch belang	Referentie
RNA metabolisme									
	SF3B1	OC	CLL	5-24		2	2 (slechte outcome, agressief, refractair aan chemo + frequent fludarabine refractair)	1	Sujobert 2018, Rosenquist 2016, Hung 2018, Bogusz 2016
	XPO1	OC	CLL	1-10		0	0	1 (XPO-inhibitor)	Sujobert 2018, Hung 2018
			PMBL	24		0	0	1 (XPO-inhibitor)	Sujobert 2018, Dubois 2016
Andere									
RNA helicase	**DDX3X**	TSG	NKTCL	20		1	1 (slechte prognose)	0	Rosenquist 2016, Zhang 2018
			BL	30		1	0	0	
Ubi-quitinatie	**FBXW7**	TSG	CLL	1-5		?	1 (slechte prognose)	?	Rodriguez-Vicente 2017

Attachment 3. Proposed ComPerMed workflow for biological classification of somatic variants, retrieved from FROYEN et al. (24)

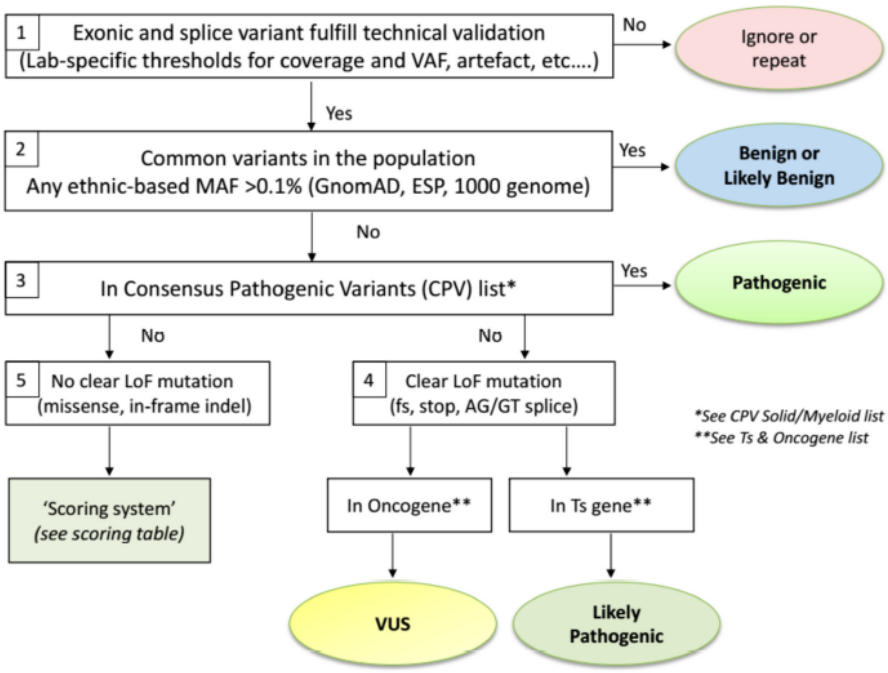


Figure 1. ComPerMed workflow for the biological classification of somatic variants.

Table 3. Scoring Table for the biological variant classification of non-loss-of-function (LoF) variants.

Parameter	Score +2	Score +1	Score +0.5	Score 0	Score −1
Total # of entries of that particular AA change at that position in COSMIC	Solid: ≥50 Hemato: ≥10	50 > x > 10 10 > x > 5	/ /	≤10 ≤5	/ /
In silico prediction tools SIFT and MutationTaster	/	/	Both damaging and deleterious	Other	/
Harmful in functional studies (PubMed, JAX-CKB, MDA, MCG)	/	/	Yes	Not reported	No
Described in at least one genomic db (CIVIC, ClinVar, OncoKb, VarSome)	/	/	As (Likely) Pathogenic	Not described/unknown	As (Likely) Benign

Variants with a score ≥2 will be classified as "Likely Pathogenic". Variants with a score <2 are classified as "VUS".