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Federaal Kenniscentrum voor de Gezondheidszorg
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Belgian Health Care Knowledge Centre

NEXT GENERATION SEQUENCING GENE PANELS FOR TARGETED THERAPY IN ONCOLOGY AND HAEMATO-ONCOLOGY

KCE REPORT 240
HEALTH TECHNOLOGY ASSESSMENT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

(a glossary is available in Appendix 1)

ABBREVIATION	DEFINITION
ACMG	American College of Medical Genetics
AFMPS	Agence Fédérale des Médicaments et des Produits de Santé
ALL	Acute lymphoblastic leucemia
AML	Acute myeloid leukemia
AMP	Association for Molecular Pathology
ASR	Analyte specific reagent
ATC	Anatomical Therapeutic Chemical
BCR	Belgian Cancer Registry
BELAC	Belgian accreditation body
cDNA	copy Deoxyribonucleic Acid
CRM	Commission for the Reimbursement of Medicines
CGH	Comparative genomic hybridisation
CAP	College of American Pathologists
CE	Conformité Européenne
CFR	Code of Federal Regulations
CISH	Chromogenic in situ hybridization
CLIA	Clinical Laboratory Improvement Act
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
DG	Directorate general
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic Acid
DOB	Date of birth
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency

EMQN	European Molecular Genetics Quality Network
EORTC	European Organisation for Research and Treatment of Cancer
EPEMED	European personalised medicine association
EQA	External quality assurance
ER	Estrogen receptor
ESP	European Society of Pathology
EU	European Union
FAGG	Federaal agentschap voor gezondheidsproducten
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence In Situ Hybridisation
FOD-VVVL	Federale overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu
GIST	Gastrointestinal stromal tumours
HER2	Human Epidermal growth factor Receptor 2
ICER	Incremental cost-effectiveness ratio
Ig	Immunoglobulin
IHC	Immunohistochemistry
IMA - AIM	Intermutualistisch Agentschap - Agence Intermutualiste
ISH	In situ hybridisation
ICD-10	International Classification of Diseases – 10th edition
ICD-O-3	International Classification of Diseases for Oncology – 3rd edition
INAMI	Institut national d'assurance maladie invalidité
INCA	Institut National de Cancer
INSS	Identification number of social security
IPH	Institute for Public Health (WIV-ISP)
ISO	International organization for standarization
IVD	In vitro diagnostic
IVDD	In vitro diagnostic directive

KC - CC	Kankercentrum – Centre du Cancer
KCE	Federal healthcare knowldege centre
LYG	Life years gained
MDS	Myelodysplastic syndrome
MLPA	Multiplex ligation-dependent probe amplification
MPN	Myeloproliferative neoplasms
MPS	Massive parallel sequencing
mRNA	Messenger RNA
PARP	Poly ADP ribose polymerase
PCR	Polymerase chain reaction
PML	Promyelocytic leukemia
PV	Polycythemia vera
NCCN	National Comprehensive Cancer Network
NGS	Next generation sequencing
NHS	National Health Service
NICE	National institute for health and care excellence
NIH	National Institutes of Health
NSCLC	Non-small cell lung cancer
PCR	Polymerase chain reaction
PMA	Pre-market approval
PML	Promyelocytic leukemia
PR	Progesteron receptor
PV	Polycythemia vera
QA	Quality assurance
QALY	Quality adjusted life year
QFI	Quantitative functional index
RAR	Retinoic acid receptor
RAS	Rat Sarcoma

RCPA	Royal College of Pathologists of Australasia
RCT	Randomised controlled trial
RD	Royal decree (KB/AR)
RIZIV	Rijksinstituut voor ziekte- en invaliditeitsverzekering
RNA	Ribonucleic Acid
ROC	Receiver operating characteristic
RT-PCR	Reverse transcriptase PCR
RUO	Research use only
SOP	Standard operating procedure
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
SPF-SSCE	Service public fédéral Santé publique, Sécurité de la Chaîne alimentaire et Environnement
TAT	Turnaround time
TCR	T cell receptor
TKI	Tyrosin kinase inhibitor
TMC	Technical Medical Council
VAT	Value added tax
WHO	World Health Organisation
WIV-ISP	Wetenschappelijk instituut voor volksgezondheid – Institut Scientifique de Santé Publique
WGS	Whole Genome Sequencing
WT	Wild type

SCIENTIFIC REPORT

1 INTRODUCTION

1.1 Background

This report on targeted therapy in oncology/haemato-oncology and the role of panel tests using next generation sequencing (NGS) is the result of a joint project of the Belgian Cancer Centre (KC-CC) and the Belgian Healthcare Knowledge Centre (KCE). A KCE project on the importance of the accuracy of companion diagnostics in routine care was joined with an evaluation of next generation sequencing panel tests in oncology and haemato-oncology. This latter evaluation was requested by RIZIV-INAMI and the pathologists/clinical biologists/geneticists and clinicians exploring this new technology. It is part of the Thematic Working Group 'Personalized Medicine' of the Cancer Centre, which was installed by the Minister of Health and Social Affairs aiming at new actions and measures in the area of personalized medicine for cancer.

More and more medicines come to the market, targeted to a specific molecular pathway that can be identified before treatment starts. Usually, a companion diagnostic is used to make sure the disease of the patient is characterised by this particular pathway, which can then be targeted. Precision medicine (also named targeted treatment or personalised medicine or stratified medicine) has been defined as identifying the right drug, for the right patient, at the right dose, at the right time.[1] The advantage of this targeted approach is that the patients treated are more likely to respond to the treatment, and that one avoids that patients who are unlikely to respond are exposed to a potentially toxic treatment. Policy makers have high expectations of targeted treatments. "Personalised medicine offers tremendous opportunities for better care and raise high expectations. If we are able to target the right patients for a medication, we reduce nonresponders and side effects, avoiding much suffering and in addition waste of money." (Commissioner Dalli's participation at the European Alliance for personalised medicine, Brussels, 2012, available from http://ec.europa.eu/commission_2010-2014/dalli/docs/speech_18092012_en.pdf). The scope of this project is restricted to in vitro diagnostics in oncology as targeted therapy in oncology is more developed compared with other therapeutic areas.

In the past, cancers used to be histopathologically defined disease entities, which were uniformly treated by dedicated therapeutic regimens. In the past decade, systemic cancer treatment moved away from this paradigm, toward the use of molecularly targeted drugs in subsets of patients, within histopathologically defined tumour types, and selected on the basis of specific genetic changes/biomarkers. When used in an appropriate way, targeted therapies are both safer and more efficacious as demonstrated by the success of trastuzumab (Herceptin®) in HER2-amplified breast cancer, imatinib (Glivec®) in chronic myelogenous leukaemia and gastrointestinal stromal tumours (GIST), gefitinib (Iressa®), erlotinib (Tarceva®) and afatinib (Giotrif®) in EGFR-mutated non-small cell lung cancer, cetuximab (Erbitux®) and panitumumab (Vectibix®) in RAS-wild type colorectal carcinoma, and vemurafenib (Zelboraf®) in BRAF-mutant melanoma.

Pivotal in the delivery of "personalized cancer medicine' are diagnostic tests, that address the presence of specific properties, 'biomarkers', of the patients' tumours: these biomarkers predict the likelihood of response to specific targeting agents (predictive biomarkers), or may provide prognostic information about the disease (prognostic biomarkers). They guide treating physicians in matching patients with available therapeutic agents ("targeted therapy") or therapeutic regimens ("tailored therapy"). Several technologies are currently in use for the diagnostic assessment of a limited number of tumour biomarkers, including immunohistochemistry (IHC), conventional cytogenetics, *in situ* hybridisation (ISH), array comparative genomic hybridisation (CGH), the polymerase chain reaction (PCR), Sanger sequencing and mass spectrometric genotyping.

It is expected that the "one drug – one companion diagnostic" combination will be replaced by a (somatic) genetic fingerprint of the tumour using next generation sequencing (NGS), also named massively parallel sequencing (MPS). This is to be completed as appropriate with germline fingerprinting (hereditary genetic characteristics of the individual), and expression profiling (mRNA, protein level).[2] The integration of all molecular data with clinical information is likely to improve treatment outcome in the near future.[3] NGS panels of (somatic) mutations covering multiple tumour types are currently being introduced in the clinic but performance requirements and interpretation of results need further standardization.[2] There is a risk of information overload for the treating physician and lack of clear guidelines on how to best use this information for patient care. Given the variability in (somatic) genetic make-up of tumours over time and by environment (e.g. metastases) it is to be envisaged that somatic fingerprinting may need to be repeated in case of resistance to treatment or for individual metastases, as targeted treatment might differ between metastases. Sampling and testing multiple metastases is currently however not yet an accepted practice. The spatial and temporal heterogeneity defining the evolution of lung cancer has been documented.[4] Peripheral blood samples ("liquid biopsies") analysed for circulating free DNA from tumour cells, are also expected to advance this field, avoiding the need for invasive biopsy procedures. "Roughly ten different major signal transduction pathways can drive tumour growth: estrogen and androgen receptor pathways (ER, AR), the developmental pathways Wnt, Notch, Hedgehog, TGFbeta, and FGF, the inflammatory NFkappaB pathway, and a number of related growth factor PI3K pathways. With the increasing availability of targeted drugs, each directed towards blocking one of these pathways, for every cancer the responsible pathway needs to be identified in order to choose the right drug (combination). The respective tumour driving signalling pathway can differ between tumours of different cellular origin, but also between primary tumours of the same kind (e.g. ER positive breast cancers), between similar tumours in different stages of progression (e.g. a primary versus a metastatic tumour), and even within one and the same tumour due to the presence of multiple cancer cell clones or differences in the microenvironment of the tumour cells. Finally, within metastatic tumours different pathways may conceivably become activated depending on organ location and associated locally defined conditions. The influence of the microenvironment on cancer cell biology and pathway activity is by now firmly established and implies that determining signalling pathway activity in a cancer sample based solely on mutation information obtained from the sequenced genome or exome of the cancer cell is often confounding, and may lead to erratic results."[5]

Targeted treatments pose challenges in terms of validation of the diagnostic criteria, especially as multiple genetic variations may co-exist and interact in a single tumour. It should be noted that NGS is currently mainly used in a clinical research setting. This can be in trials with new targeted drugs or in rescue treatment trials in heavily pre-treated patients where the identified mutations guide the off-label use of existing targeted drugs. This may lead to an extension of indications of the existing targeted drug. However, an increase in the off-label use is more likely to be seen. Indeed this phenomenon of increased use of targeted drugs off-label has already been observed in routine care in case the NGS panel test identifies a mutation in a gene that would otherwise not have been interrogated. (personal communication L. Van Kempen). Any potential health effects or the possible budget impact of increased off-label use of targeted drugs are however not well documented.

Table 1 provides a list of targeted anticancer drugs (ATC code L01XC for monoclonal antibodies and L01XE for small molecules, tyrosine kinase inhibitors) reimbursed in Belgium. The technique to detect the biomarker is sometimes specified in the reimbursement criteria but often the technique is mentioned in a quite generic manner like for example “molecular biological technique” without further specification. Also note that NGS currently does not offer a detection platform for all molecular companion diagnostics in use in oncology. Detection of gene amplifications (e.g. HER2) with NGS or translocations (e.g. ALK) with RNA NGS are however being studied. The list is based on drugs for which a companion diagnostic is required in the reimbursement criteria. For other drugs the companion diagnostic use is not specified as a reimbursement condition, e.g. all-trans retinoic acid (Vesanoid) is used in those cases of acute promyelocytic leukemia showing a chromosomal translocation of chromosomes 15 and 17, which causes genetic fusion of the retinoic acid receptor (RAR) gene to the promyelocytic leukemia (PML) gene.

Table 1 – Targeted therapies reimbursed in Belgium (December 2014)

Diagnostic marker(s)	Technique	Indication	Targeted therapy
HER2	ISH	Breast cancer	Herceptin (trastuzumab)
HER2	ISH	Breast cancer	Herceptin SC (trastuzumab)
HER2	ISH	Breast cancer	Tyverb (lapatinib)
HER2	ISH	Breast cancer	Perjeta (pertuzumab)
HER2	ISH	Breast cancer	Kadcyla (trastuzumab-emtansine)
HER2	ISH	Stomach cancer	Herceptin (trastuzumab)
BCR-ABL	cytogenetics or PCR	Chronic Myeloid Leukaemia	Glivec (imatinib)
BCR-ABL	cytogenetics or PCR	Chronic Myeloid Leukaemia	Sprycel (dasatinib)
BCR-ABL	cytogenetics or PCR	Chronic Myeloid Leukaemia	Tasigna (nilotinib)
BCR-ABL (not T315I nor V299L)	cytogenetics or PCR (?)	Chronic Myeloid Leukemia	Bosulif (bozutinib)
Kit (CD117)	IHC	Gastrointestinal stromal tumour	Glivec (imatinib)
Kit (CD117), no *PDGFRA* D842V mutation	IHC and ?	Gastrointestinal stromal tumour	Glivec (imatinib)
CD20	IHC	Non-Hodgkin-lymphoma	MabThera (rituximab)
CD20	IHC	Follicular lymphoma	Zevalin (ibritumomab)
KRAS wt and NRAS wt	?	Colorectal cancer	Erbitux (cetuximab)
KRAS wt and NRAS wt	?	Colorectal cancer	Vectibix (panitumumab)
EGFR	IHC	Non-small-cell lung carcinoma	Tarceva (erlotinib)
EGFR-TK activating mutation	?	Non-small-cell lung carcinoma	Tarceva (erlotinib)
EGFR-TK activating mutation	?	Non-small-cell lung carcinoma	Iressa (gefitinib)
EGFR-TK activating mutation	?	Non-small-cell lung carcinoma	Giotrif (afatinib)
ALK	IHC and FISH	Non-small-cell lung carcinoma	Xalkori (crizotinib)
BRAF V600 mutation	?	Melanoma	Zelboraf (vemurafenib)
BRAF V600 mutation	?	Melanoma	Tafinlar (dabrafenib)
EpCam	IHC	Malignant ascites	Removab (catumaxomab)

?=technique not specified in the reimbursement criteria other than molecular biology method (currently mainly PCR/sanger sequencing). Note that currently only BCR-ABL T315I, V299L, PDGFRA, KRAS, NRAS, EGFR-TK activating mutation and BRAF V600 tests can be performed with NGS panel tests.

In KCE report 20 (2005) on molecular diagnostics in Belgium,[6] it was recommended to require ISO15189 accreditation for molecular tests, assuming this would lead to centralisation and high quality. The purpose was that the oncology handbook in each hospital should serve as guidance for the sequential and appropriate use of specific molecular tests in oncology. It was recommended that laboratories performing molecular tests for oncology should offer the full panel for a given tumour.[6] Service level agreements between hospitals should facilitate that tests are conducted outside of the own hospital. Article 33bis of the nomenclature of reimbursed activities was introduced following the KCE report, indeed requiring accreditation according to the ISO15189-standard for medical laboratories.

Despite a clear improvement in quality, it seems that obtaining ISO accreditation from BELAC is achieved by most laboratories who apply for accreditation, illustrating that an upgrade in quality took place among medical laboratories, but this implicates that the aim of centralisation was not realised. Over 20 laboratories in Belgium received BELAC ISO15189-accreditation for the local molecular testing in oncology.

Another recommendation in KCE report 20[6] concerned the need for a common reimbursement review of the drug and companion diagnostic at RIZIV/INAMI. A change in the review process to include information on predictive markers of targeted drugs was implemented recently. (https://www.riziv.fgov.be/webprd/appl/pssp/SSP/DEM2/Pdf/GuideClass1nl.pdf) This project also aims to identify requirements and possible process changes such that the expected benefit of targeted therapy for the patient can be realised since the test reimbursement criteria lag behind the clinical practice and are not easily modifiable. A look to the experience abroad for interaction of diagnostic and therapeutic regulations and reimbursement may be of help in this regard. In addition, the system should be able to cope with the fast evolution in molecular testing technology.

The overall objective of this project is to find options to introduce NGS panel tests for somatic mutations in oncology/hematology in the Belgian healthcare system. The focus in this project is on NGS panels as an alternative technique for the currently accepted and well documented molecular tests for selected somatic genetic changes in solid tumours and in haematological malignancies. This focus was requested by RIZIV-INAMI and the pathologists/clinical biologists/geneticists and clinicians exploring this new technology. The project scope includes markers for patient selection (positive or negative) for targeted therapy (the companion diagnostics), but also markers for diagnosis confirmation (primary tumour or metastasis), and markers for prognosis. Markers retained for reimbursement should have clinical management implications as detailed in clinical practice guidelines or practice-changing publications. As the technical standardisation and clinical utility of this technology still needs further study, such innovations are ideally covered in a research setting. The aim of this project is to present the current clinical utility of NGS gene panel tests and to define the requirements that should be met before such tests enter the routine care.

A second aim of this project is to describe the importance of the accuracy of the companion diagnostic in targeted therapy in oncology. Differences in diagnostic accuracy between the tests used in phase 3 trials versus the tests used in routine clinical practice can significantly impact on patient benefit, harms and the cost-effectiveness of the treatment, which is mainly based on phase 3 trial results. Comparative effectiveness research that takes into account the routine test accuracy is not yet fully developed. Also the impact of the introduction of NGS panel tests will be discussed in this respect.

1.2 Research questions

The following research questions are addressed:

1. What are the indications for NGS panel testing in oncology/haematology and what should be the characteristics of such panels (composition with level of clinical utility, technical specifications, informed consent and reporting specifications, quality assurance,..) such that the technology can be implemented in routine clinical care, as an alternative method for the currently accepted single gene markers?.
2. What is the added value of NGS panel tests when compared with current practices; what is the cost for performing NGS panel tests?
3. What is the impact of the diagnostic accuracy of the companion diagnostic on the cost-effectiveness of the treatment from a healthcare payer perspective?
4. How are targeted therapy and companion diagnostics reimbursed in Belgium (and abroad)?

5. What could be options for financing this technology during a transitional period and which further data could be collected during such a period, further supporting its clinical use?

1.3 General approach

To address the above questions, KCE and CC have created this project and called on support to the major stakeholders in the field. The project was managed by F. Hulstaert (KCE) and M. Van den Bulcke (KC-CC) and supported by a Steering Group and dedicated working groups that included domain experts to document the desired panel composition, the required quality assurance measures, the current testing activity in Belgium, the cost aspects and options for further data collection during a transient research financing phase. The Steering Group included representatives of the RIZIV-INAMI, FOD-VVVL/SPF-SSCE, FAGG/AFMPS, BELAC, WIV-ISP, the College of Oncology, the College of Human Genetics, the Commissions of Pathology and Clinical biology specialists as well as the rapporteurs of the working groups who drafted the respective chapters of this report: P Vandenberghe and S Tejpar for the composition of NGS panels; E Dequecker, R Salgado, J Van Houdt, and S Van Laere for the chapter on quality; B. Maes for the table showing the use of the billing codes by tumor type, L San Miguel for the health economic analyses on the importance of the test accuracy; H. De Schutter for the analysis of billing code volumes by tumor type; A Waeytens for the RIZIV-INAMI reimbursement procedures for targeted treatment, and the four coded laboratories willing to report their NGS cost analysis. Within the short time frame of this project no systematic review of the clinical utility of the markers listed was undertaken.

Further details of the literature search used or other methods are listed at the start of each chapter.

1.4 Evaluation of diagnostic tests

Fryback and Thornbury have described a 6-level hierarchy for evaluating diagnostic tests.[7] A diagnostic test does not necessarily need to have demonstrated effectiveness at each level before it can be used in clinical practice. However, this approach clearly presents the possible gain and remaining uncertainty on the test's efficacy. This framework has been detailed in a previous KCE report and used in pilot assessments of a number of molecular diagnostic tests,[6] and could be used to evaluate NGS panel tests in oncology.

Level 1: technical efficacy

The technical efficacy of a test refers to the ability to produce usable information. The *test's feasibility* and *operator dependence* refer to in what circumstances and by whom the test can be performed. The *analytical sensitivity* is the ability of a test to detect a target analyte (the measured component), which is usually expressed as the minimum detectable concentration of the analyte. This should be distinguished from the diagnostic sensitivity, the ability of a test to detect disease. The precision or *reproducibility* of results is the ability to obtain the same test results on repeated testing or observations. It is influenced by analytical variability and observer interpretation. Analytical variability consists of inaccuracy and imprecision. Inaccuracy implies systematic error, such as calibration error. Imprecision implies random error.

Level 2: diagnostic accuracy

This level refers to the test's ability to detect or exclude disease in patients compared with a criterion standard or reference test. Test characteristics are sensitivity, specificity, predictive values, likelihood ratios and receiver operating characteristics (ROC) curves. *Sensitivity and specificity* are the most widely used outcome measures, but are sensible to spectrum bias. Spectrum bias may occur when the study population has a different clinical spectrum (more advanced cases, for instance) than the population in whom the test is to be applied. For example, if sensitivity is determined in seriously diseased subjects and specificity in clearly healthy subjects, both will be grossly overestimated relative to practical situations where diseased and healthy subjects cannot be clinically distinguished in advance.

Predictive values, with the positive predictive value being the proportion of patients with a positive test result that actually has the disease and the negative predictive value the proportion of patients with a negative test result that does not have the disease, are dependent on disease prevalence in the study sample. The *likelihood ratios* show how a test result alters the pre-test probability into a post-test probability, using Bayesian reasoning. The pre-test probability depends on the prevalence of the target condition and the results of previous tests, for example history, clinical examination, imaging

or laboratory tests. Another outcome measure which is sometimes used, is the *number needed to diagnose*, analogous to the number needed to treat in treatment intervention studies. However, using this measure it is assumed that diagnostic testing is always done to rule in a target condition, to diagnose the target condition, while in clinical practice tests are also used to rule out a target condition.

Finally, test accuracy can be illustrated using an *ROC curve*. The ROC curve plots test sensitivity versus 1-specificity for various cut-off points. The area under the curve provides a summary measure of the test performance. It also allows to compare two different tests based on their respective area under the curve or partial area under the curve in which the test is most useful.

Clearly, the first level of diagnostic efficacy, technical efficacy, contributes to the diagnostic accuracy. But it also becomes apparent that there may be a point beyond which improvement in technical performance no longer improves diagnostic accuracy. Assuming therefore that diagnostic accuracy can be estimated on the basis of technical accuracy studies is not correct.

Level 3: diagnostic thinking

This level of diagnostic efficacy is concerned with assessment of the effect of test information on diagnostic reasoning and disease categorization. Studies on diagnostic thinking serve as a proxy for estimating the effect of a test on patient care. Patients' outcome cannot be influenced by the diagnostic technology unless the physician is acting differently than would have been the case without the test information.

Using the *likelihood ratio* and calculating the post-test probability, this change in diagnostic thinking can be computed. However, the pre-test probability of a disease is not always available in clinical practice and depends not only on setting, but also on patient characteristics and other selection processes, such as referral and the results of previous tests. Clinicians who wish to apply the Bayesian properties of diagnostic tests require accurate estimates of the pre-test probability of target disorders in their disease area and setting (e.g. primary care versus specialist care).

An alternative are studies that empirically test the *change in the physician's subjective assessment* on the probability of disease. In these studies, physicians are asked to estimate the probability of disease before knowing the test result, and to estimate it again after the test result has been disclosed. Efficacious tests are those that significantly increase or lower pre-test probabilities assumed by the physician or computed by likelihood ratios using Bayesian reasoning.

One major difficulty with this level of diagnostic efficacy is that it is not always known what post-test probability of disease should be used as a threshold. Which probability of disease is low enough to exclude disease, which is high enough to treat the patient?

Level 4: therapeutic impact

The most efficacious tests at this level are those that lead to the institution of a new management strategy. Studies can assess this empirically by comparing the intended management before the test result is known with that after the test result has been disclosed. In what *proportion of patients did the information change the intended management*? In some cases, management changes are considered not only in the patient himself, but also in other persons, for example prophylactic measures in case of an infectious outbreak. These prospective case-series, however, can be subject to bias such as selection bias. The lack of a concurrent control group may lead to confounding, as there is no information on those patients not enrolled in the study and therefore not receiving the new technology. These considerations underscore the need for randomized controlled trials (RCTs). But, in the absence of RCT's they do play an important role as an intermediate.

Level 5: patient outcome

The ultimate goal of health care is to improve patient outcome. For diagnostic tests that are expensive, potentially harmful or widely used, knowledge about patient outcome efficacy seems particularly important. It is at this level that expected harm, such as burden, pain, risk, can be weighed directly against its expected benefit, such as improving life expectancy, quality of life, avoiding other test procedures, etcetera.

The *randomized controlled trial* is the study design the least prone to bias to estimate these harm and benefit. However, it is not always feasible to perform an RCT for ethical, financial or other reasons. In those cases, case-series collected before and after the introduction of a new test technology or case-control studies may provide some of the answers.

A methodological difficulty with this level is that the independent contribution of test technology to patient outcomes may be small in the context of all the other influences and therefore very large sample sizes may be required. But, in spite of these difficulties, RCT's on diagnostic tests are feasible.

Some tests, however, will never be able to prove a change in 'objective' patient outcomes such as mortality or morbidity, simply because there is no treatment available at this moment that has an impact on these outcomes. A diagnostic test will therefore never produce a difference in mortality, but may improve *quality of life measures* by giving the patient (and the carer) an affirmative diagnosis and providing an explanation for the signs and symptoms the patient experiences.

Level 6: cost-effectiveness analysis

This level goes beyond the individual risks and benefits, but assesses whether the cost for use of a given test is acceptable for society. Is the price for the positive effect on patient outcome worthwhile? Resources cannot be allocated twice; money spent on one technology cannot be spent on another.

Cost-effectiveness studies compute a cost per unit of output. Any of the measures of the previous levels can be used as input, for example cost per surgery avoided, cost per appropriately treated patient, cost per life year gained or cost per quality adjusted life year (QALY) gained. Final outcomes, such as life years gained or QALYs gained, are preferred over intermediate outcomes in economic evaluations, as they allow comparisons across a broader range of health interventions, e.g. diagnostic and therapeutic interventions. Because data on these outcomes and costs of the diagnostic and subsequent therapeutic paths are not routinely available from observations, modelling becomes inevitable to examine the cost-effectiveness of diagnostic tests. The validity of the model input parameters is crucial for the credibility of the model. The values of all input variables must be based on solid evidence obtained from literature or observations. Sensitivity analyses can illustrate the robustness of the conclusions, by demonstrating the sensitivity of the results to changes in the values of those input variables that remain uncertain.

2 TOWARDS NEXT GENERATION SEQUENCING PANEL TESTS IN ONCOLOGY

2.1 Molecular diagnostics used in oncology

Here, we briefly summarize the range of methods available for molecular diagnosis, their relative value for detecting genomic variation and some key technical challenges for each technology when applied in a clinical setting.

It should be indicated that different types of test offerings coexist today: tests for use in the hospital based on IVD kits or in-house developed tests, and tests that are commercially provided, often using a limited number of commercial centralized testing facilities.

Each method is briefly discussed below.

The polymerase chain reaction (PCR): from here to eternity

The polymerase chain reaction (PCR) is a biomedical technology used to amplify a piece of DNA across several orders of magnitude, generating millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, after which the method is named, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations and is today still one of the key molecular diagnostics in medical practice.

PCR is commonly applied and accepted in the clinical setting today, being relatively cheap and highly sensitive and specfic. Disadvantages are the need for thorough development and tedious validation as limited post-reaction verification is possible unless additional analyses are performed (e.g. sequencing).

Fluorescent in situ hybridisation (FISH): detection of structural and chromosomal variation

Recent improvements in chemistry and microscopy have substantially augmented the resolution of cytogenetics, most notably through the development of multi-probe fluorescent in situ hybridization (FISH) and chromosomal comparative genomic hybridization (CGH). Cytogenetic methods are now sometimes replaced/complemented by combined SNP–array CGH approach that uses probes to detect chromosomal and genomic rearrangements as well as deletions with greater precision and smaller genomic variations than FISH.

Multiplex ligation-dependent probe amplification (MLPA): a cost-effective solution for complex questions

Multiplex ligation-dependent probe amplification (MLPA) is a rapid, high-throughput technique for copy number quantification and methylation status analysis of genomic sequences. MLPA is a variation of the multiplex polymerase chain reaction that permits multiple targets to be amplified with only a single primer pair. Each probe consists of two oligonucleotides which recognize adjacent target sites on the DNA. One probe oligonucleotide contains the sequence recognised by the forward primer, the other the sequence recognised by the reverse primer. Only when both probe oligonucleotides are hybridised to their respective targets, can they be ligated into a complete probe. Each complete probe has a unique length, so that its resulting amplicons can be separated and identified by (capillary) electrophoresis. This avoids the resolution limitations of multiplex PCR. Because the forward primer used for probe amplification is fluorescently labeled, each amplicon generates a fluorescent peak which can be detected by a capillary sequencer. Comparing the peak pattern obtained on a given sample with that obtained on various reference samples, the relative quantity of each amplicon can be determined. This ratio is a measure for the ratio in which the target sequence is present in the sample DNA.

MLPA has a variety of applications in congenital and hereditary disorders and in tumour profiling, including detection of mutations and single nucleotide polymorphisms, analysis of DNA methylation, relative mRNA quantification, chromosomal characterisation of cell lines and tissue samples, detection of gene copy number, detection of duplications and deletions in human cancer predisposition genes such as BRCA1, BRCA2, hMLH1 and hMSH2.

Genome-wide single nucleotide polymorphism (SNP) microarrays: simple, cheap, fast but valid?

Microarray-based genotyping can be divided into three main applications: array comparative genomic hybridisation (array CGH) to detect structural anomalies, phenotype-specific SNP panels, and genome-wide SNP panels.

Efforts in academic and commercial laboratories have produced phenotype-specific panels containing alleles that are known to drive many specific phenotypes. The utility of this approach is that a low-cost, expeditious experiment interrogating multiple genes can offer high-quality molecular diagnoses. However, the continuous discovery of novel causal alleles and genes, as well as variable penetrance and expressivity of known mutations limits the clinical validity/utility of this approach.

By contrast, large-scale genome-wide SNP genotyping offers a single, cost-efficient platform to assess risk of multiple common genetic disorders with variably documented associations in one test. Predictive and pre-symptomatic testing is available as a multiplex platform for a host of conditions, including certain cancers and pharmaco-genetic tests.

Sanger DNA sequencing: today's gold standard?

For detection of point mutations and small variants, bidirectional Sanger sequencing has been considered the 'gold standard' in clinical genetic testing for the past decade but has a significant lower sensitivity for detection of lower frequency variants than NGS. This direct approach has high analytical validity, although long reads can deteriorate quality for base calling, and minute specimens can produce PCR artefacts. The fundamental value in directly sequencing one or more entire genes is the ability to combine a clinical indication for a candidate gene with the high sensitivity and specificity of the assay.

Whole genome sequencing (WGS): the future promise?

Cost considerations notwithstanding, the primary practical barrier to the use of WGS in clinical settings is the limited ability of the technology to reliably detect the absence or presence of mutations. Different sequencing platforms and bioinformatics pipelines have been shown to deliver results of variable quality, with some being more accurate at individual base calls and others covering a broader range of the genome.[8]

Targeted approaches, including specific gene panels (or in some cases also exome-sequencing) may be of greater analytical sensitivity having a better coverage of the target to detect heterozygous changes, but restrict the clinical sensitivity in comparison to WGS, which might limit the interpretive scope to coding lesions.

At present it is not yet possible to obtain a high-quality sequence for the entire human genome.

Targeted next generation sequencing (NGS): one door, one key facilitator

NGS has the major advantage that with less material, more useful information can be obtained. The technology has become affordable for routine use but demands a major logistic and training effort. It is discussed in the next section.

2.2 Next generation sequencing, the technology

Various NGS platforms exist. They employ different technologies but their underlying workflow is similar.[9]

1. **Fragmentation of DNA**. For NGS panel tests in oncology the fragment length should not be too short (also PCR based methods need sufficiently long DNA fragments)
2. **Ligation to adaptor sequences**. These are platform-specific sequences that are ligated to the ends of DNA fragments, creating the sequencing library. Alternatively strategies without fragmentation and ligation exist for producing sequencing libraries, for example a multiplex PCR with primers that include the platform specific adaptor sequences.
3. **Immobilisation**. This occurs through the adaptor sequence to a solid surface, such as a bead or a glass slide.
4. **Clonal amplification**. Most platforms require amplification to increase the signal for detection. This can be achieved through emulsion bead PCR or surface cluster PCR.
5. **Sequencing**. Cycles of base incorporation by synthesis or ligation are followed immediately by signal detection. Signals are converted to base calls, from which a nucleotide sequence or 'read' is produced. The read length is about 200 bp for NGS panel tests in oncology, which is shorter than Sanger sequencing reads, which may be 800 to 1000 bp. Each template DNA region is also sequenced a number of times (depth of coverage).
6. **Data analysis**. Due to the short read lengths, there is limited ability to reassemble a genome through overlapping sequences. Most applications of NGS involve 'resequencing', where sequencing is used to search for variations from 'normal' using a known reference genome to provide a template for read alignment. Differences between the alignment and reference sequence are identified ('variant calling'), filtered and annotated to identify those that may be clinically significant.

Currently two NGS platforms are mainly used in Belgium

Illumina (MiSeq/NextSeq/HiSeq) performs sequencing by synthesis.

"The DNA (sequencing) library is immobilised onto a lane of a flow cell, consisting of a glass surface coated with millions of primers complementary to the ligated adaptor sequences. Each library fragment is amplified by 'bridge-PCR', whereby different reagent washes initiate annealing of the free adaptor end with a complementary primer, extension by DNA polymerase, then denaturation of the double stranded 'bridge'. Through repeating this cycle, a 'cluster' of thousands of copies of each library fragment is created, with up to 10 million clusters per square centimetre on a flow cell. Sequencing is initiated by the addition of fluorescent reversible terminator nucleotides, DNA polymerase and universal sequencing primers. In each cycle, only a single base is incorporated, a laser excites the fluorophore and the emitted light is detected by a camera. A cleavage reagent is added to remove the fluorophore and terminator, allowing the next cycle of nucleotide extension and detection."[9]

Life Technologies (Ion Torrent Ion Proton/Ion PGM) performs sequencing by monitoring pH

"The sequencing library is amplified on capture beads by emulsion PCR. Beads now coated with amplified template are put onto a chip containing up to 7 million wells, each able to accommodate a single coated bead. In each sequencing cycle, reagent containing one of the four possible nucleotides is washed over the chip and, if the base is incorporated, there is release of a proton. The change in pH (~0.02 pH units per base incorporation) within a well is detected by the ion sensor and converted to a digital signal. In successive cycles, the different nucleotides are washed over the chip in a set order to produce a read."[9]

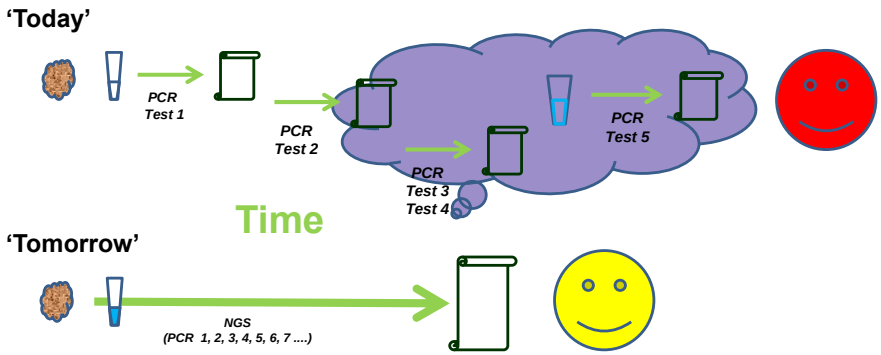
Added value of NGS in oncology/hematology diagnosis

Multiplexing with NGS may be necessary in order to save tissue and resources for example for tests in melanoma (NRAS, BRAF, cKIT), colorectal cancer (NRAS, KRAS, BRAF). The growing number of molecular tests that need to be performed in a sequential manner on a single tissue sample poses problems for certain tumour types that invariably are associated with small amounts of tissue, non-small cell lung cancer biopsies being an illustrative example. This means preparing the tissue to perform an EGFR-mutation test, whereas the remaining tissue on the formalin-fixed paraffin-embedded (FFPE)-block is then prepared to perform ALK FISH. Sometimes there is no tissue left to test for ROS1 FISH. Performing an additional biopsy in patients with cancer is sometimes needed, which is associated with potential complications. This sequential approach also has an effect on the overall turnaround time (TAT) of the results, which is the totalling of the TAT of each sequential analysis.

In many haematological malignancies a wide variety of recurrently mutated genes have been described. While some can be conveniently detected by RT-PCR or DNA-PCR, others are not fit for diagnostic PCR, due to widespread nature of mutations throughout the coding region of the gene and the absence of mutational hot spots. In addition, several of the recurrently mutated genes recur only at low frequency, indicating the need to test for a large gene set.

A targeted multi-testing approach may therefore be cost-saving since within one and the same run multiple parameters are assessed, combined with a lower turnaround time and a more optimal utilisation of the tissues. This is illustrated in figure 1 below

Figure 1 – Advantages and disadvantages of NGS compared with other molecular diagnostics



Advantages:	- ***Less*** material for ***more*** info - Info at the DNA sequence level (***precision*** higher) - Parallel analysis, ***faster*** conclusive results
Disadvantages:	- ***New*** paradigm - Major primary ***investment*** - ***Complex*** interpretation - Does ***not substitute*** for all molecular testing (e.g. translocations)

Errors and quality scores in NGS

"Errors and biases can be introduced during any of the steps involved in NGS. The different technologies are all prone to different sequencing errors to varying degrees. For example, the Ion PGM has difficulty accurately sequencing homopolymers greater than 8 bases long, while the Illumina MiSeq has difficulties with GC-rich motifs. Base calling errors are estimated by a base call quality score (Qscore)."[9]

"If p is the estimated error probability, Qscore = −10.log10(p). For example, if the error probability of a base call is 1/100, the base call Qscore is 20. Base calling algorithms are platform specific, and convert the signal detected to a base call with a Qscore. The probability of base call errors depends on parameters such as the signal to noise ratio, cluster/bead density and dephasing (loss of sequence cycle synchronicity due to incomplete or extra extension). These errors may be better identified (and therefore not be translated into errors in variant calling) by increasing depth of coverage, replicate sequencing or sequencing across different platforms, but this increased volume of sequencing may significantly increase overall costs. Due to these limitations, Sanger sequencing, which has a lower raw error rate, is still considered the 'gold standard' and used to confirm variants identified through MPS."[9] The concept of "golden standard" is however evolving. It is possible that in the future any orthogonal (different) method can be used to confirm NGS-findings or that identified alterations do not need to be confirmedl.[10]

False positives and false negatives

"The overall error rate is a determinant of the sensitivity and specificity for detecting low frequency variants. This is often the case when identifying somatic changes in cancers, where samples may be heterogeneous due to low amount of tumour tissue vs normal tissue or presence of multiple tumour subclones. A 30X depth of coverage is generally considered sufficient to identify SNPs, while for cancer genomes it may need to be much higher (500–1000X), depending on the mutation frequency and platform used.

Incomplete coverage, where regions are not sequenced or poorly sequenced, is also a problem seen with all MPS technologies. This may be due to amplification or enrichment biases and inherent difficulty in sequencing particularly GC-rich and GC-poor regions. It may be necessary to perform Sanger sequencing to fill-in these gaps in coverage and completely sequence areas of interest."[9] The nuances mentioned above should also be considered.

"Both false positive and false negative variant calls may be made due to errors in alignment, alignment with an unsuitable reference sequence or too stringent or lenient filtering. Correct annotation is highly dependent on the accuracy of information obtained from interrogated databases, such as those containing known disease-causing mutations, common polymorphisms or mutations in cancer."[9]

2.3 Introduction to next generation sequencing panel tests

2.3.1 The sequencing revolution

In the last few years, the advent of next generation sequencing (NGS) technologies has contributed to changing the way cancer is perceived. Using this comprehensive "massively parallel" technology, multiple cancer types have been studied in the quest for genomic alterations that could provide predictive, diagnostic or prognostic information as illustrated below. The level and type of evidence of the different genes in a gene panel and the interpretation for eventual clinical use can be categorized as illustrated below.

Table 2 – Predictive, diagnostic and prognostic genomic alterations and level of evidence, adapted from Good et al.[11]

Evidence property	Evidence sub-property	Description	Example
Type of evidence	Predictive	Genomic alteration is predictive of response to therapy	Breast cancer cell lines with H1047R mutation showed increased sensitivity to CH5132799 compared to cells with wild-type PIK3CA gene BCR-ABL1 fusions type b2/b3a2 are sensitive to imatinib and other TKI
	Diagnostic	Genomic alteration is diagnostic for disease or subtype	DNAJB1:PRKACA fusions are very strongly associated with the fibrolamellar variant of liver cancer BCR-ABL1 fusions type b2/b3a2 are diagnostic for chronic myelogenous leukemia
	Prognostic	Genomic alteration is prognostic for disease outcome	The presence of RUNX1/RUNX1T1 of CBFB-MYH11 fusions in acute myelogenous leukemia is prognostically favourable The presence of FLT3 ITD is unfavourable in AML
Level of evidence	A - validated association	Proven/consensus association in human medicine	A meta-analysis of clinical studies showed that harboring a BRAF V600E mutation predicts worse prognosis in patients with colorectal cancer
	B - clinical evidence	Clinical trial or other primary patient data supports association	In non-small-cell lung cancer patients with EGFR T790M and other activating mutations, their progression-free survival is shorter than those who do not have T790M mutations
	C - preclinical evidence	In vivo or in vitro models support association	Experiments showed that AG1296 is effective in triggering apoptosis in cells with the FLT3 internal tandem repeat
	D - inferential association	Indirect evidence	Glioma cells harboring IDH1 mutation may be more susceptible to chemotherapy or radiotherapy due to their reduced ability to respond to oxidative stress

Note: The schema for evidence types and levels was inspired by Van Allen et al.[12].

Somatic alterations associated with cancer were identified using whole genome and exome sequencing. In the same manner, gene arrangements such as fusion genes and translocations conferring sensitivity to selective therapies in specific cancer types can be found in multiple cancer types. Indeed, ALK and ROS 1 rearrangements sensitizing non-small cell lung cancer (NSCLC) to ALK inhibitors are also present in subtypes of colorectal cancer.[13] In breast cancer, putative driver mutations were identified in several new cancer genes including AKT2, ARID1B, CASP8, CDKN1B, MAP3K1, MAP3K13, NCOR1, SMARCD1 and TBX3.[14] In addition, studies identifying mutations or aberrations that can predict response to standard therapies are emerging but not yet fully integrated in a health care setting as clinical validation is still ongoing. In luminal breast cancer for instance, the presence of GATA3 gene mutations appeared to be correlated with aromatase inhibitor induced anti-proliferative effects in the neoadjuvant setting,[15] but so far GATA3 is not yet part of the armamentarium of any laboratory offering assays to treating physicians.

The same paradigm shift is taking place in haematology. While a number of cytogenetic and molecular aberrations have been known since the beginning of this century a remarkable effort has been underway to identify the genetic basis of hematological malignancies catalyzed by increasing availability and more refined sequencing technologies. This has led to the discovery of a completely novel mutational landscape of of recurrently mutated genes or pathways in acute myeloid leukemia,[16] acute lymphoblastic leukemias, mature lymphoid neoplasms and chronic myeloproliferative neoplasms, and myelodysplastic syndromes. In hairy cell leukaemia, whole exome sequencing identified somatic mutations in five genes: BRAF, CSMD3, SLC5A1, CNTN6 and OR8J1. Another 47 cases were screened for BRAF mutations and strikingly, the BRAF V600E substitution was found in all 47 patients evaluated. This finding was translated into clinical benefit with the treatment of hairy cell leukaemia patients with a BRAF inhibitor (vemurafenib) currently approved for melanoma.[17, 18] A phase II trial is currently ongoing and enrolling patients (NCT01711632). A MYD88 L265P-activating mutation was recently found in 90% of Waldenstrom's macroglobulinemia cases. Another major discovery in acute myeloid leukaemia was the identification of mutations in IDH1, which encodes isocitrate dehydrogenase 1, and the related IDH2 gene.

Other alterations affecting the splicing machinery, transcription and translation, have been uncovered. The next step is to translate these discoveries into drug discovery following the example of BCR-ABL in chronic myeloid leukaemia on the one hand and on the other hand to translate these findings in a health care model.

2.3.2 *Importance of targeted sequencing for exploratory clinical research in oncology*

Centres involved in clinical research could use NGS panel tests also as a tool to identify patients eligible for clinical trials using targeted drugs. For targeted therapy trials, many pharmaceutical trials will only recruit centres where cancer patients had their tumour already characterized with a (broad enough) NGS panel test. This accelerates and facilitates the identification of eligible patients and limits the costs and time of centralized companion diagnostic testing. This strategic advantage is to be seen in a highly competitive clinical research environment with significant research and economic implications for the concerned larger hospitals and their staff. These drugs may be existing targeted drugs not (yet) developed for the indication under study. Some examples of such trials are given below, with a focus on patient outcome.

A personalized medicine program for patients referred to the Phase I Clinic was initiated in 2007 at The University of Texas MD Anderson Cancer Center and molecular testing was requested for 1 283 patients. Metastatic biopsies were not mandatory and molecular profiling was conducted via PCR-based sequencing technology. At least one molecular aberration was identified in 460 out of 1 144 samples and patients were treated with matched therapy when available or with standard phase 1 treatment if no aberration was identified or no matched therapy was available. Treatment assignment was not randomized. In patients with one alteration, matched therapy (n = 143) compared with treatment without matching (n = 236) was associated with a higher objective response rate (12% vs. 5%), longer PFS (median, 3.9 vs. 2.2 months), and longer survival (median, 11.4 vs. 8.6 months). In multivariate analysis, matched therapy was an independent factor predicting response ($P < 0.015$) and PFS ($P < 0.004$).[19]

Another large experience is the Institut Gustave Roussy MOSCATO 01 (molecular screening for cancer treatment optimization) program. Patients undergo a metastatic lesion biopsy and NGS is performed before treatment assignment. Interim analyses were presented after the enrollment of 129 heavily pretreated metastatic cancer patients. 86% of patients had a metastatic lesion biopsy and an "actionable" aberration was detected in 40% of cases. 23% of patients were treated with matched therapy and response rate was 20% which is quite high in the phase I setting.[20]

CUSTOM is a fully reported prospective trial enrolling 668 patients with NSCLC, small cell lung cancer (SCLC), and thymic cancer. Patients were assigned to different treatment groups based on genetic mutations or amplification detected using next-generation sequencing of almost 200 genes. The trial demonstrated the feasibility of performing extensive genetic testing on a tumour biopsy in a timely manner-in this case, taking only 2 weeks to complete. It also highlighted the safety of new biopsies from patients with advanced cancer providing the tissue needed for the analysis.[21]

Now, the SAFIR02 program (NCT01414933) - a multicentric phase II randomized trial, using high throughput genome analysis as a therapeutic decision tool, comparing a targeted treatment (administered according to the identified molecular anomaly of the tumour) with a chemotherapy administered without considering the tumour genome analysis - aims at demonstrating the medical benefits of a genomic analysis based treatment in breast cancer.

In Belgium, two pilot trials were initiated and completed in order to prove the clinical feasibility and utility of NGS in patients with metastatic breast cancer (Breast International Group pilot trial: NCT02094742) and metastatic melanoma, colorectal cancer and NSCLC (Precision-f: NCT01932489).

A large European initiative, AURORA (Aiming to Understand the Molecular Aberrations in Metastatic Breast Cancer NCT02102165) has started enrolling patients with metastatic breast cancer and the first recruiting centres are in Belgium and Luxembourg. The goal of this program is to understand metastatic breast cancer while previous initiatives had focused on primary breast cancer.[22] Another important goal is to design downstream genomic-driven clinical trials in order to assign patients to targeted treatment. Indeed, new clinical trial designs are another challenge in the era of NGS. "Actionable" alterations are rare such as HER2 mutations that are present in 2 to 3 % of metastatic breast cancer.

New cancer subtypes based on molecular screening instead of histology are transforming common cancers such as NSCLC into "orphan" diseases. Adaptive designs such as in I-SPY2 (Neoadjuvant and Personalized Adaptive Novel Agents to Treat Breast Cancer NCT01042379), "basket" trials such as with vemurafenib (A Study of Zelboraf, vemurafenib, in Patients With BRAF V600 Mutation-Positive Cancers NCT01524978) and "umbrella" trials such as Lung-MAP in squamous NSCLC (Lung-MAP: S1400 Biomarker-Targeted Second-Line Therapy in Treating Patients With Recurrent Stage IIIB-IV Squamous Cell Lung Cancer NCT02154490) are necessary in order to move new drug discovery forward.

"Liquid biopsies" are another promising technology that could help patients avoid sequential biopsies and also play a role in the discovery of drug resistance mechanisms and in relapse prevention.

Proving clinical utility for many of these markers is not straightforward if not linked to a formal drug development program. Four different levels of acceptance of biomarkers can be identified:

Level 1: well accepted biomarkers with proven clinical utility, in routine use

Level 2: biomarkers used in ongoing clinical trials phase 2/3

Level 3: known biomarkers but clinical significance not known, perhaps some preclinical data

Level 4: newly identified biomarkers

Table 2 and table 3 show different examples of possible classifications of clinical utility. Table 3 illustrates the complexity using a categorization that is more focused on the clinical management consequences for the patient. The level of evidence decreases from A to C in Table 3.

Additional initiatives, some in collaboration with the pharmaceutical industry, are given below:

- Targeted therapy in metastatic cancer in The Netherlands (http://www.cpct.nl/)
- Targeted therapy in lung cancer in UK and US:
- In the UK, patients with non-small cell lung cancer (NSCLC) can participate in a nationwide trial (MATRIX), a collaboration of the NHS with some pharmaceutical companies. The aim is to treat the patients with marketed on label, but also off-label and non-marketed drugs, based on the molecular profile of the tumour as assessed using NGS.

  http://www.cancerresearchuk.org/about-us/cancer-news/press-release/2014-04-17-revolutionary-clinical-trial-aims-to-advance-lung-cancer-treatment-thanks-to-cancer-research-uk-and

  http://scienceblog.cancerresearchuk.org/2014/04/17/stratified-medicine-and-the-lung-cancer-matrix-trial-part-of-a-cancer-care-revolution/

  http://www.focr.org/lung-map
- A similar approach is being used at the European Organisation for the Treatment of Cancer (EORTC) to enroll colon or lung cancer patient into the clinical trial that is most appropriate based on the tumour NGS fingerprint (the SPECTA platform). http://www.eortc.org/taxonomy/news-categories/specta. NGS is performed at the Wellcome Trust Sanger Institute.

Table 3 – Example of classification of clinical utility of molecular alterations, copied from André et al, 2014[23]

Level of evidence	A	B	C	Clinical implications
I: Molecular alteration validated in several robust early phase trials or at least one phase III randomized trials	Alteration validated in the disease under consideration, targeted therapies have shown to be uneffective in patients who are lacking the genomic alteration	No evidence that the therapy does not work in the absence of the molecular alteration	Level I molecular alteration, but not in the disease under consideration	A/B: Patients must be treated with the targeted therapy C: Patients should be considered for clinical trials
II: Efficacy of targeting molecular alteration suggested in single and underpowered phase I/II trials	Alteration validated in the disease under consideration, targeted therapies have shown to be ineffective in patients who are lacking the genomic alteration	No evidence that the therapy does not work in the absence of the molecular alteration	Level I molecular alteration, but not in the disease under consideration or anecdotal evidence of response to targeting molecular alteration in single patient case reports	Patients should be considered for clinical trials testing the targeted therapy
III: Target suggested by preclinical studies	Preclinical studies include human samples, cell lines and animal models	Preclinical studies that lack either cell lines or animal models	NA	Inclusion in clinical trials is optional
IV: Target predicted but lack of clinical or preclinical data	Genomic alteration is a known cancer-related gene	Genomic alteration is not known as cancer-related gene	NA	Inclusion in clinical trials is optional

Source : Annals of Oncology (Oxford University Press): Prioritizing targets for precision cancer medicin (F.Andre, E. Mardis, M. Salm, J.-C. Soria, L.L. Siu, C. Swanton), 12 Jan 2014; 25;12

2.3.3 New technologies, more options, new challenges

The NGS technology has enabled worldwide collaborative research efforts, aiming at the comprehensive genetic and epigenetic characterization of those cancer types with the highest global incidence and mortality. Besides the identification of several novel actionable cancer genes in several malignancies, this initiative is also revealing an unexpected complexity of the mutational landscape of several malignancies, and of the patterns underpinning disease phenotype, prognosis, treatment response and resistance. Moreover, there is an unanticipated somatic genetic heterogeneity within histopathologically defined tumour types, and even within single patients, intratumoural heterogeneity is common. As a result, the cancer field is moving away from the binary simplicity of biomarkers and early personalized medicine towards what is now called "precision medicine".

The implementation of NGS is expected to lead to improved detection of therapeutic targets, of markers of prognostic value, or of rare or uncommon alterations to be studied in clinical trials. Embracing this novel genetic technology and translating it to the clinical cancer diagnostic laboratory can therefore expand treatment options for individual patients. In addition, NGS will likely also facilitate the personalized follow-up of minimal residual disease.

An important requirement for this paradigm shift to happen would be the translation of massive parallel sequencing of cancers from the research setting to the clinical diagnostic setting, especially as it relates to the identification of mutated genes that can be 'targeted' using either small molecule inhibitors or specific antibodies. Development of the necessary infrastructure to ensure that genetic testing data are properly handled and utilized in clinical settings is also an important issue for the realization of cancer precision medicine. Ideally, the adoption of this new technique in clinical practice in various countries should occur in an internationally coordinated manner.

In the USA, targeted NGS is already reshaping the care of the cancer patient. Experts envision a requirement of widespread clinical implementation (including larger community hospitals) within 2 to 3 years. Optimized NGS will detect a broader range of genomic alterations than current clinical assays. Recent data have shown that only colorectal cancers without mutations of KRAS and NRAS exons 2-4 benefit from EGFR directed therapies while patients/tumours harboring such mutations may be harmed. NGS panel tests offer the possibility to test for all of these mutations already today.

NGS panel tests can be performed in a laboratory of a hospital or can be offered as a commercial service whereby the sample is shipped to the central facility, often abroad.

2.4 Composition of the NGS Panels

Methodology

As this information needs to be updated rather frequently, the composition of specific NGS panels is to be seen as a living database. The presence of a marker listed under Appendix 2 of this report is to be merely taken at this stage as informative and cannot be considered in whatsoever context as being endorsed by the KCE, the Cancer Centre or the Belgian government for any clinical use.

In order to focus the effort on introducing NGS as a tool in molecular profiling of tumors towards clinical utility, the working groups were asked to draw up a first list of targets that could have diagnostic, prognostic and predictive clinical value. It was decided that separate lists would be compiled for solid and haematological (myeloid and lymfoid separately) tumors. Where available, key references were already included. These three lists were then 1°) discussed at consecutive working group meetings and 2°) compared with targets currently included in commercial cancer panels of Illumina and Ion Torrent. Reagent kits are marketed for research use only (RUO) or have a CE mark (see 3.1.2 for regulatory context). None of the oncology NGS panel tests currently has FDA market approval. All five lists were then sorted alphabetically and occurrence of the respective targets in the respective lists scored. Then, the targets were listed according to their common occurrence in the five lists). The latter list was designated the 'Be-NGS gene panel' list.

In addition, the 'Be-NGS gene panel' list was compared to two other lists that became available during the project. First, a recently compiled cancer marker gene list, "The Cancer Genomics Resource List 2014", published by Zutter M. et al. (2014), for the College of American Pathologists (CAP). This publication and the annexes are available online for consultation. (http://www.archivesofpathology.org/doi/abs/10.5858/arpa.2014-0330-CP).

Second, a list of cancer target genes compiled by a team of French cancer experts under supervision of the 'Institut National de Cancer' (INCA, France) (see Appendix 2 Table A1).

Using the 'Be-NGS gene panel' list, a survey was sent to all members of the working group . It was requested to complete the list with information on the clinical use of each marker (predisposition, prognostic, diagnostic, prediction) and on the use of different technologies (FISH, PCR, NGS) to detect somatic mutations /gene amplifications/ gene rearrangements in these tumor markers. Eight responses were received by 12 december 2014, of which seven were used to document the current opinion of the participants on the proposed 'Be-NGS gene panel' list (see Appendix 2 Table A2).

Summary of the results

Together, these analyses show that 1°) a concensus on the clinical utility of a small set of target genes exists amongst different teams of experts and that most of these targets are also present as such in the commercial NGS gene panels of Illumina and Ion Torrent, 2°) a considerable number of targets proposed by the Belgian expert group are either not withheld at all, either withheld by only a limited number of other expert teams, 3°) the opionion on the clinical utility of the different withheld target differs between the responding Belgian experts.

A systematic review of the clinical utility of the different target within an NGS cancer gene panel is beyond the scope of this study. The above results do however stress the need for further collaborations within the Belgian context on a common approach in the use of such molecular technologies in cancer medicine and urge concertation with international knowledge support initiatives on tumor profiling.

2.4.1 NGS panels for solid tumours

Several approaches are available, each with unique advantages and disadvantages. For solid tumours, gene panels for targeted resequencing are commercially available, or will be available soon, from Multiplicom®, Illumina®, Agilent® (Haloplex®), Qiagen® and Life Technologies®, such as the Cancer Research Panel (Haloplex®), the Ion AmpliSeq Cancer Hotspot Panel (Life Technologies®), the Trusight Tumour (TST) panel and the TruSeq Amplicon Cancer Panel (TSACP) (Illumina®), and the MASTR kits (Multiplicom®). The kit product inserts state that they can be used starting from FFPE materials, and this claim remains to be validated. More extensive panels, covering more than 90 genes are also available from Life Technologies® and Illumina®. Clinical oncologists tend to favor limited gene panels (restricted to actionable mutations) or intermediate size gene panels (including non-actionable recurrent or prognostic mutations as well). At the present time, extensive gene panels appear less well suited for a clinical diagnostic purpose than for research and discovery.

Arguments in favor of commercial standard panels, in comparison with home-designs, are their immediate availability, their prior documentation (for example with regard to a more uniform coverage), and their wider user community. However, also commercial IVD kits require sufficient validation by the laboratory that wants to use the kit. Their cost is lower than that of custom kits by the same manufacturers, which are tested only *in silico*. However, some commercial kits are exclusively designed for a specific sequencing platform, and may thus be less suitable based on the local platform choices. Finally, the match of the gene panel with the local needs can be less than optimal.

As can be seen from the panel compositions in the Appendix 2, the commercial gene panels show extensive overlap, and cover many cancer genes relevant for lung, colorectal, breast, thyroid and brain tumours, and melanoma, GIST and gynecological malignancies. Many laboratories opt for a single design or a few panels for solid tumours. There is variation in the spectrum of genes and mutations covered in the panels. The kits that are currently offered by Multiplicom®, or to be introduced soon, cover only 20 genes but offer the advantage of having a CE-label, reflecting previous validation and a reduced batch-to-batch variability. On the other hand, between 40 and 50 genes are addressed by cancer panels by Illumina TSACP®, Haloplex® (Agilent) or Life Technologies®, while the Illumina TST® panel takes an intermediate position. While the panels marketed by Illumina and Life Technologies are specifically designed for their own sequencing platforms, respectively the Illumina (HiSeq 2000-2500 and Miseq) or the Life Technologies IonTorrent platforms, the independent manufacturers Agilent and Multiplicom provide kits which are adapted to either platform.

2.4.2 NGS panels for hemato-oncology

First, as many hematological samples are directly processed from EDTA or heparin preserved liquid samples, without fixation, there is no concern about FFPE-related artifacts, except for lymph node biopsy specimens. Second, the spectrum of mutations in hematological malignancies is wide, distinct from the solid tumour spectrum, and not covered by commercially available panels. This has started to change in the course of 2014, with the introduction of several commercial panels covering the mutant genes in myeloid disorders such as acute myeloid leukemia (AML), polycythemia vera (PV), essential thrombocythemia (ET), myelofibrosis (MF), chronic myelomonocytic leukemia (CMML). Yet, at the time of writing no such panels are available to our knowledge for lymphoid neoplasms, including the acute lymphoblastic leukemias and the mature lymphoid neoplasms (non-Hodgkin lymphoma, NHL) Therefore, development of a customized gene panel design relevant for the spectrum of hematological malignancies may be required based on a consensus which will have to be reached. Criteria for inclusion into this gene panel will be the incidence of concerned gene mutations in the hematological malignancies to be covered, their value for diagnosis, disease classification, disease prognosis, or their potential as an actionable therapeutic target. A tentative list for acute leukemias, chronic myeloproliferative neoplasms, myelodysplastic syndromes and mature lymphoid neoplasms is proposed in Appendix 2, based on literature data and supported by emerging consensus panels. It remains to be seen whether it will be more appropriate to run myeloid and lymphoid panels separately versus as one panel.

Concerning custom panels, several companies offer kits for the preparation of custom amplicon libraries: Illumina®, Life Technologies®, as well as Agilent® (Haloplex®), however, based on *in silico* data only.

In general, implementing NGS-testing in a clinical setting emphasizes a multidisciplinary approach, whereby the choice of the panel size and the panel composition should align with the institutional strengths and opportunities that the hospital can offer to patients. There is no doubt that centres with a strong academic and clinical trial setting may opt for a wider gene panel approach so to be able to identify patients for reimbursable agents, but also for use of off-label treatments and identification of patients eligible for ongoing clinical trials. It is emphasized though that the most important actionable variants should be part of each panel.

2.4.3 Other considerations for NGS panel selection

The costs associated with performing a full panel of for example 50 genes is only moderately higher compared with a more limited panel (see chapter on cost). Defining the full comprehensive panel for the whole of Belgium may not be possible, but at least each panel should contain the minimum set of actionable variants that are to be detected under good clinical practice. Evidence-based clinical practice guidelines, when updated frequently enough, should mention the relevant molecular alterations to be detected in the context of good clinical practice.[24, 25]

This minimum set of actionable genetic alterations should be detailed and updated regularly and be detected with a highly similar sensitivity and specificity in all centres, whatever the nature of the centre is. In this context it should be mentioned that currently different panels, from different providers or home-made, tested on different platforms may not completely have overlapping sequenced regions. This is not acceptable from a good clinical practice perspective and requires further standardization such that one avoids rendering different results on the same case when analyzed in different laboratories.

Key Points

- **The NGS technology provides new opportunities to realise targeted therapy**
- **The different steps involved in NGS panel tests need further standardization, both for use in routine care and in clinical trials.**

3 REGULATORY ASPECTS, QUALITY ASSURANCE AND EDUCATIONAL NEEDS

3.1 Somatic versus hereditary mutations

The focus of this report is on somatic mutations, not hereditary mutations. Whereas somatic mutations that characterize a tumor have no direct hereditary character, this is different for germline mutations that may be discovered simultaneously when the NGS panel test is run. Such unsollicited genetic screening is to be avoided.The assumption used in this report is that the NGS panels used to detect somatic mutations do not interrogate important hereditary markers. This has also been realized in centres offering NGS panel tests in routine care abroad.

However, this situation may change quickly. BRCA1/2 mutated or dysfunctional cells may be sensitive to poly ADP ribose polymerase (PARP) inhibition by synthetic lethality. EMA recently recommended the marketing authorisation for the PARP inhibitor Lynparza for treatment of ovarian, fallopian tube and primary peritoneal cancers in women with BRCA mutations. Somatic BRCA1- and 2-mutations will thus have to be included in gene panels of daily practice for the treatment selection of serous high grade ovarian cancer.[26, 27] Pre-test genetic counselling of ovarian cancer patients therefore seems necessary when such panel test would be offered in the future. The logistics that will be needed if this principle would also become standard practice for more common types of cancer needs a separate study and is out of scope of this report.

3.2 Regulatory context of molecular tests for somatic mutations

3.2.1 Regulations for diagnostic laboratories in Belgium

Licensing – EQA selection and control by WIV-ISP

All diagnostic laboratories, clinical biology, pathology and genetics, need to be licensed in Belgium in order to obtain the reimbursement of routine tests as specified in the RIZIV-INAMI nomenclature. For clinical biology laboratories the requirements for licensing were published in RD 3rd of December 1999 which required the implementation of a quality management system according to the 'praktijkrichtlijn van de commissie van klinische biologie'. A comparable regulation for licensing the laboratories of pathology was published in 2011 (RD on the recognition of pathology laboratories 5th December 2011) and became mandatory in the 1st of March 2013. In this RD it is mentioned that a quality management system needs to be implemented according to the 'praktijkrichtlijn van de commissie van pathologie'. For both types of laboratories the Scientific Institute of Public Health (ISP-WIV) is responsible for the compliance to the requirements of the license to practice. The WIV-ISP defines and controls the EQA scheme participation of laboratories of clinical biology and pathology in the context of obtaining and maintaining their license.

The EQA schedules are imposed by the respective commissions for clinical biology and pathology and the elaboration, evaluation and follow-up is executed by the WIV-ISP. Participation in these schedules by these laboratories is mandatory for obtaining and maintaining their license.

For molecular testing, there is one and the same EQA scheme for all participating Belgian laboratories of clinical biology and pathology, organized by the WIV-ISP. Today, these EQA schemes of WIV-ISP cover only a limited fraction of the molecular pathology tests in oncology and haemato-oncology.

ISO 15189 – EQA free selection, audited by BELAC

The requirements mentioned in the RD of 14th December 1987 for licensing of centres of human genetics were updated with additional requirements, in 2012. Since the 1st of January 2014 all genetics laboratories need to be accredited according to ISO 15189 and this for 80% of the activities they perform (art33 of the Belgian nomenclature). The requirement of ISO 15189 accreditation also applies for some tests in the field of molecular haematology and oncology (art33bis of the nomenclature). The ISO 15189 accreditation is granted by BELAC and is required in addition to the licensing for reimbursement. So far a medical laboratory in Belgium can only be accredited with a fix scope. Each changes in method or adding new gene(s) in a panel of a diagnostic test, the laboratory needs to ask a new accreditation visit before they can implement it in the scope. Given that oncology and hemato-oncology tests in Belgium need to be accredited (art 33bis) this give a delay in implemented new developments. In other countries the accreditation bodies works with a flexible scope in medical labs which give the opportunity to the labs to implement in a more efficient way new developments in diagnostic tests.

The EQA participation needed in the context of the ISO 15189 accreditation is currently not controlled nor standardized by the WIV-ISP. The laboratories can decide themselves which (ISO accredited) EQA scheme(s) fits their needs to support their accredidation. According to the experience of the experts, some EQA schemes are more difficult to pass than others. The EQA participation and the results are checked during the yearly BELAC audit.

In summary, all laboratories in Belgium performing diagnostic tests need to operate according to a quality system, including all steps starting from collection and reception of the samples (pre-analytical), analysing the samples as well as interpretation and reporting of the results.

One of the aims of EQA is the inter-laboratory comparison of the Belgian laboratories. Therefore, there may be a need for streamlining the selection, control and reporting of a common and appropriate EQA programme in a transparent way by WIV-ISP and BELAC.

3.2.2 Regulations for in-house methods and in-vitro diagnostics

The European in vitro diagnostic directive 98/79/EC (IVDD) became operational in June 2000. (http://www.qarad.com/data/acms/docs/f58eb519593748d185456480e10d32c3/1_revision_of_europes_ivd_directive.pdf) It provides Europe with one single regulation for IVD devices. Devices receiving the CE mark after one successful conformity assessment procedure can be introduced into the member states of the European Union and the European Free Trade Association, Switzerland and Turkey —32 European countries in all.

In contrast to the US Food and Drug Administration (FDA) which requires demonstration of safety and effectiveness of devices, the European system requires the pre-market evaluation of safety and 'performance' of a device. The term 'performance' is however not defined.

More than 10 years of implementation also revealed weaknesses in the IVDD. For example, there is a consensus that its classification system is inadequate. Furthermore, scientific and technological evolutions, as well as new business trends in the IVD field - for example, the emergence of companies offering IVD testing as a commercial service - created situations that are not effectively foreseen in the IVDD. A revision of the IVDD was needed to eliminate the weaknesses of the current version and to provide a regulatory framework within which issues arising from both technological progress and new business trends can be addressed.

In the current IVDD, in-house testing ("home brew") is excluded from the scope, if the "devices are manufactured and used only within the same health institution and on the premises in the immediate vicinity without having been transferred to another legal entity." This is often misinterpreted by companies offering diagnostic services, from within the EU or from outside. They often erroneously conclude that their in-house testing activities are also exempt from CE-marking. However, preamble 11 and article 9(13) of the IVDD clearly bring commercial IVD testing within the directive's scope. One may expect that the revised IVDD will clarify which in-house testing is exempt from the directive and which is not. The Public Consultation document suggests that the European regulators also are considering restricting the exemption to certain types of in-house testing, e.g., tests for rare diseases or tests in lower Global Harmonization Task Force classes. The obligation for the testing laboratory to have an accredited

quality system—in accordance with ISO15189, for example—or for the tests themselves to meet the directive's essential and/or other requirements are other options that may be considered. In addition to emphasizing or clarifying that diagnostic services are subject to the IVDD, specific requirements may be defined. These include requirements related to advertising and the provision of information to patients and users, especially when these companies directly advertise and communicate results to users. It will be interesting to see how the revised IVDD will address the fact that increasing numbers of tests are offered, sometimes directly to the consumers, without the tests ever being physically placed on the market, and whether any such measures can be effectively enforced on companies that are not located within the EU but nevertheless offer their services to European consumers. The inclusion of offering a test for an EU citizen while he or she is within the EU into the definition of "placing a product on the market" is an option.

Genetic tests (definition not clear) that have a medical purpose are already covered by the current version of the IVDD. However, the medical purpose of a genetic test is not always clear, such as with predictive tests and lifestyle tests. The European Commission clearly intends to clarify the situation, and the Public Consultation primarily asked how this could be achieved.

As expressed in a statement of the European Society of Human Genetics, "all laboratories offering genetic testing services should implement an internal quality system and be subject to regular external quality assessment"[28] Accreditation of the tests offered is the standard for guaranteeing the quality of molecular genetic testing for health purposes. All persons involved in the provision of genetic services (i.e. medical doctors, nurses, genetic counselors, and biologists and technicians working at the laboratories) should have the appropriate qualifications and training and perform their role in accordance with professional best practices and ethical standards".[28]

Within the current European regulatory context, there is little emphasis on clinical evidence. It is hardly mentioned in the IVDD. In practice, there is much more focus on analytical performance than on clinical evidence. European intended-use statements and performance characteristics are often expressed in analytical terms. Conformity assessment procedures, with or without a notified body, can be done with relatively little attention to the clinical validity of the test results. This is probably one of the major differences between the European and U.S. regulatory systems for IVDs and devices in general.[29] Whereas the European directives require the demonstration of safety and performance (a non-defined term), the FDA requires the demonstration of safety and clinical effectiveness for really new devices (under the pre-market approval procedure).[29]

In the US, a distinction is made between IVDs and ASRs. An IVD, in vitro diagnostic, is cleared or approved by the FDA for one or more specific intended uses with established analytical and clinical performance characteristics. FDA approval refers to products that are approved for marketing under the PMA (pre-market approval) process for new devices. FDA clearance refers to devices that are cleared for marketing under a 510 (K) review. Generally the PMA process is more stringent, targeting truly novel products. Devices that are conceptually similar to those already on the market, or that represent improvements over existing products, can elect FDA clearance under a 510(K).

ASRs, analyte specific reagents, are products for use in "home-brew" testing, which have manufacturer assurances of GMP. ASRs must be labeled in accordance with 21 CFR § 809.10(e). Advertising and promotional materials are regulated by 21 CFR § 809.30(D). The laboratory that develops an in-house test using the ASRs shall inform the ordering person of the test result by appending to the test report this statement according to 21 CFR § 809.30(e): "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the U.S. FDA." ASRs do not have any intended use claims; the laboratory is responsible for establishing intended use and cutoffs. In the US, RUO (research use only) tests are not intended for use as building blocks for laboratory-developed assays and cannot be used for clinical laboratory testing.

As an illustration and not directly applicable to the NGS panel tests in oncology and haemato-oncology, the FDA authorized for marketing four next generation sequencing based diagnostic devices which consisted of two heritable disease-specific assays, library preparation reagents and a NGS platform that are intended for human germline targeted sequencing from whole blood. These examples clearly illustrate the different

methodological difficulties that are encountered when willing to apply a "classical" IVD-approach to this technology, namely

1. An NGS-assay has the potential to query an almost unlimited number of variants, some of which have unknown significance, rendering not only analytical validation, but also clinical validation difficult for these variants;
2. As mentioned previously, there is no appropriate reference method that defines the "truth" when performing accuracy studies. The premarket authorization of the cystic fibrosis clinical sequencing assay by FDA illustrates particular considerations to this approach, namely:
   a. The necessity to validate accuracy across the entire region of the gene being claimed,
   b. The necessity to validate the specific mutation types across relevant gene regions (either specific indels or the largest insertion or deletions claimed);
   c. The necessity to demonstrate assay performance in problem regions such as areas with high GC content, homopolymeric regions and areas with high homology to other regions of the genome and finally
   d. The necessity to include all relevant mutations of known clinical consequence.[30]

The MiSeqDx was required to meet the following FDA expectations for specimen and processing-related validation:

1. The specimen type(s) as source of nucleic acid;
2. The type(s) of nucleic acids (e.g. germline DA, tumor DNA) and the nucleic acid extraction method.

On the other hand the following requirements on sequencing variation-related validation should be complied with:

1. Type(s of sequence variations (e.g. single nucleotide variations (SNV), insertions, and deletions);
2. Type(s) of sequencing (e.g. targeted sequencing);
3. The read depth required for the sensitivity being claimed and the validation data that supports the claim;
4. Accuracy and precision of the test and the types of sequence variations that the test cannot detect with the claimed accuracy and precision (e.g. insertions or deletions larger than a certain size, translocations) and
5. The upper and lower limit of input nucleic acid to achieve the claimed accuracy and reproducibility.[2]

3.3 External quality assurance of molecular tests for somatic mutations

3.3.1 EQA schemes for Haematological and Solid Tumours

External quality assessment (EQA) is defined by the World Health Organization as "a system of objectively checking laboratory results by means of an external agency" (External quality assessment of health laboratories: report on a WHO Working Group, 1981, WHO: Copenhagen, http://whqlibdoc.who.int/euro/r&s/EURO_R&S_36.pdf consulted December 2014). The diagnostic accuracy in a routine care situation may be different from the highly standardized and centralized testing in RCTs. Having a look at the results of EQA schemes for such tests, e.g. HER2 FISH, may provide some insights into possible issues in this regard. This is of relevance as in the next chapter the importance of the diagnostic accuracy of companion diagnostics for the cost-effectiveness of the targeted treatment will be assessed.

Participation in external quality assessment is a requirement for ISO 15189:2012 accreditation (International Organization for Standardization. ISO 15189:2012 Medical laboratories - Particular requirements for quality and competence. ISO, Geneva, 2012) and in addition in Belgium it is a governmental requirement for reimbursement of molecular oncology tests (RD of 2007, changing the RD of 14 September 1984 concerning the nomenclature of reimbursed activities).

Because a wide variety of methods can be used for mutation analysis, it is highly important that laboratories can verify the robustness and accuracy of their method. EQA programs aim to monitor laboratory performance, allow inter-laboratory comparison, and deliver support to assure that laboratories that perform these tests provide results according to accepted predefined standards of quality.[31]

In general an EQA scheme is organized by a multidisciplinary team, consisting of medical experts, technical experts and the EQA provider team. The process of an EQA is as follows: a large number of laboratories are provided with the same material. Laboratories analyse these samples and submit their results (analysis results as well as written clinical reports) to the coordinating centre. Results are evaluated by a team of assessors which are experts in the field. At the end of an EQA scheme, each participant receives a general report with the genotypes results as well the interpretation scores, individual feedback from the assessors and a certificate of participation.

According to ISO 15189 an EQA scheme must reflect the diagnostic and clinical reality as closely as possible and it is highly recommended to cover the entire test process, including the analytical phase as well as pre- and post-examination procedures.

There are several EQA providers who work on a regional, national, or international basis for molecular oncology and hematology. In Europe, the European Society of Pathology (ESP, http://www.esp-pathology.org, last accessed January 13, 2014), the European Molecular Genetics Quality Network, (EMQN, http://www.emqn.org/emqn/Home, last accessed October 30, 2014) and the United Kingdom National External Quality Assessment Service (UKNEQAS, http://www.ukneqas.org.uk, last accessed October 30, 2014) are well-known. Their process slightly differs, the ESP and EMQN organize an EQA of ten samples once a year and UKNEQAS sends three times three samples each year. In the United States, CAP (College of American Pathologists, http://www.cap.org, last accessed October 30, 2014) is the main provider of EQA. They send three samples, two times a year. The sample material among the providers is similar, formalin-fixed paraffin-embedded material, synthetic samples or a combination of both.

3.3.2 Performance of the participating (Belgian) laboratories

3.3.2.1 EQA schemes organized by the Belgian IPH - Performance of the Belgian laboratories

For the Belgian medical laboratories of pathology and clinical biology, participation in the EQA schemes organized by the Belgian Scientific Institute of Public Health (IPH) is mandatory to obtain reimbursement for routine testing as specified by the Belgian nomenclature.

Additional ISO 15189 accreditation is mandatory to obtain reimbursement for tests performed by molecular biological techniques, as described in art. 33bis and art 33 of the Belgian nomenclature.

A summary of the EQA results of the Belgian laboratories obtained during the period 2009 -2013 is presented below. It is important to note that up to now no specific NGS panel EQA for oncology or haemato-oncology has been performed. However, all EQA results for IHC/molecular tests used as a companion diagnostic for targeted therapy were considered of relevance as these data are an indication of test accuracy in routine use (versus phase 3 trial central lab). As discussed further in the report, the cost-effectiveness consequences are important if the routine test accuracy differs from the trial setting.

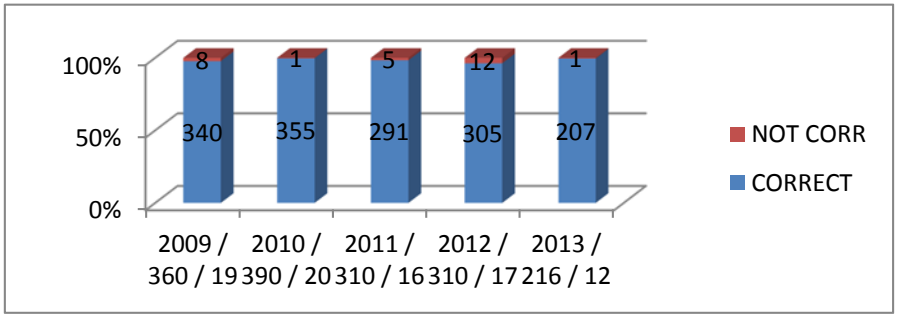
EQA HER2 (FISH) 2009- 2013

Amplification or overexpression of the oncogene HER2 plays a role in the development of breast cancer and is predictive for the response to HER2-targeted treatment. The reimbursement of this parameter was introduced by the Belgian government in 2007 (nomenclature art. 33 bis). ISO 15189 accreditation for this parameter became mandatory in 2009.

For the Belgian laboratories, an external quality assessment (EQA) scheme was organized by the Scientific Institute of Public Health –Quality of Medical Laboratories in 2009, in collaboration with the College of American Pathologists (CAP). The EQA surveys are organized twice per year, each consisting of two slides (one stained/one non stained slide) of ten different biopsies.

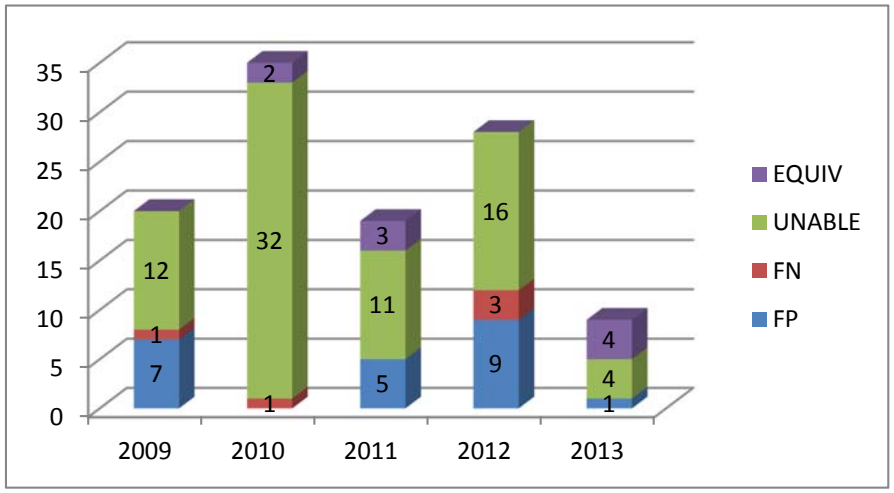
Results are shown in the graphs below:

Figure 2 – Results for the EQA of HER2 (FISH) 2009 – 2013 (Year / Nb results/ Nb labs), excluding equivocal and 'unable to analyse' results.



CORRECT: correct results – NOT CORR: false positive / false negative results
Starting in 2010, some laboratories have replaced the FISH method by another ISH method. The presented results were only for FISH methods explaining the decreasing number of participants after 2010.

Figure 3 – EQA HER2 FISH 2009-2013, detailed representation of results not classified as "correct".



EQUIV: equivocal results – UNABLE: unable to analyse - FN: false positive – FP: false negative results

EQA KRAS 2012 - 2013

The KRAS mutation status helps the physicians in identifying patients with metastatic colorectal cancer with respect to treatment. If the test result indicates that KRAS mutations are absent in the colorectal cancer cells, then the patient may be considered for treatment with anti-EGFR inhibitors. The reimbursement of this parameter was introduced by the Belgian government in 2011. (nomenclature art. 33 bis). ISO 15189 accreditation for this parameter became mandatory in 2013. For the Belgian laboratories, an external quality assessment (EQA) scheme was organized by the Scientific Institute of Public Health – Quality of Medical Laboratories in 2012, in collaboration with the College of American Pathologists (CAP). The EQA surveys are organized twice per year, one sample per survey, each sample consisting of a 10µm tissue section to be analysed for KRAS mutation status. Eight Belgian laboratories participated in the survey; for 2012 and 2013, 26/31 (83.9%) correct results were obtained, 5/31 results were considered 'acceptable' (see table below).

Table 4 – EQA KRAS Results 2012-2013

RESULTS	Nb. LABS (BE) 2012		Nb. LABS (BE) 2013	COMMENT
C.35G>C (codon 12 GGT > GCT	12/15	*(80 %)*	14/16 *(87.5%)*	Good
Mutation positive – not specified	2/15	*(13.3%)*	2/16 (12.5%)	Acceptable
Mutation positive – codon 12	1/15	(6.7 %)	---	Acceptable

** Acceptable: mutation has been detected but not fully characterized (CAP criteria www.cap.org).*

EQA HEMATOLOGY – ONCOLOGY 2009 - 2010

Reimbursement of parameters for molecular diagnosis of haematological malignancies was introduced by the Belgian government in 2009 (nomenclature art. 33 bis). ISO 15189 accreditation for this parameter became mandatory in 2009. For the Belgian laboratories, an external quality assessment (EQA) scheme was organized by the Scientific Institute of Public Health – Quality of Medical Laboratories in 2009, in collaboration with UK-NEQAS. During the period 2009-2010, the IPH had access to the results of the Belgian laboratories. From 2011, it was is difficult to obtain the individual data of the Belgian laboratories. As in Belgium ISO 15189 is mandatory to obtain reimbursement for these parameters, the survey of the quality of the Belgian laboratories is indirectly obtained by the audit reports from BELAC.

Five different EQA surveys are organized per year, two sample for each parameter. Results are shown in the table below.

Table 5 – EQA haematology-oncology results 2009-2010

Parameter	Nb Labs (BE) 2009	Correct results 2009	Nb Labs (BE) 2010	Correct results 2010
JAK2 – V617F	17	34/34	17	34/34
IGH / TCR clonal	14	27/28	14	27/29
BCR-ABL *(Quantitative)*	16	60/64	16	55/62(1)
CHIMERISM	9	16/18	9	24/26
Acute myeloid leukemia				
BCR-ABL *(Qualitative)*(+)	15	29/30(2)	16	32/32
Inv(16)-t(8;21)-t(15;17) (3)	11	67/67	11	90/90

(1): 2 missing result, 2 not detected, 5 quantitative incorrect (>+3sd), - (2): 1 missing result – (3) 12 participants for t(15;17) in last survey

3.3.2.2 European EQA programs - performance of the participating (Belgium) laboratories

The European Society of Pathology (ESP) has established a European external quality assessment (EQA) network that includes programs for molecular testing in colorectal cancer (CRC) and in non-small cell lung cancer (NSCLC).[32, 33]

Results of the ESP Colon EQA scheme of 2012 were presented recently in the Journal of molecular diagnostics.[34] Genotyping errors consisted of false positives and false negatives as well as incorrectly identified mutations. In the entire scheme (1050 cases), 9 false positives and 29 false negatives occurred. Ten cases were assigned a positive mutational status, but with an incorrect mutation reported. Thus, in total 48/1050 samples (4.57%) were incorrectly identified.

Sixteen out of 29 false negatives were assigned for a challenging sample with only 10% neoplastic cell content. Eighteen percent of laboratories made a genotyping error for this specimen and 12% reported insufficient sensitivity of the test for reliable mutation detection. Inclusion of this sample with borderline acceptable characteristics, allowed to unravel the limited

sensitivity at low neoplastic cell percentages and even more important the lack of awareness of this shortcoming in the interpretation of the result.

Twenty-eight out of 105 laboratories (27%) made genotyping errors. Eighteen laboratories (17%) genotyped one out of ten samples wrong. Errors were due to both analytical and data transcription errors. No indication for unsatisfactory performance of a specific method was observed. The table below gives an overview of participants and results of the different organized Colon ESP schemes by ESP, with a focus on Belgian participants. The results of the ESP Colon 2013 were reported recently.(personal communication E. Dequecker) At laboratory level, 26.7% made one or more mistakes. In comparison to previous schemes, the error rate is similar to the first full Colon EQA scheme in 2010 (error rate 30%).[35] In 2011 this error rate was 18.5% and in 2012 it was 26.7%, but this was mainly due to a sample with only 10% neoplastic cells to challenge the laboratories.[34] Without this sample, the error rate would have been 12.4%. The high error rate of the present scheme indicates that many labs have difficulties with extending their routine clinical testing. Probably a new learning phase is ongoing and the labs need to adjust to the new routine.

Table 6 – Overview of participants and results of the ESP Colon EQA schemes

ESP Colon EQA			
Scheme	Number of participating laboratories	Number of participating countries	Number (%) of labs with all genotypes correct (100%)
2009	61	9	42 (68.9%)
2010	76	11	51 (67.1%)
2011	124	27	89 (71.8%)
2012	105	26	79 (75.2%)
2013	131	30	51 (38.9%)

ESP Colon EQA - Belgium			
Scheme	Number of Belgian laboratories	Mean genotype score (%)	Number (%) of Belgian labs with all genotypes correct (100%)
2009	10	87	5 (50.0%)
2010	13	95	5 (38.5%)
2011	12	94	8 (66.7%)
2012	12	97	11 (91.7%)
2013	16	93	5 (31.3%)

A wide variety of methods can be used for mutation analysis. Table 7 gives information on the methods used by all laboratories to identify mutation in the *KRAS* and *NRAS* gene for exons 2,3 and 4. The kit based tests used in this EQA program were mostly CE IVD labeled CE-IVD (108 labs, 94%) or for research use only (7 labs, 6.1%). The 3rd column reflects the situation of the Belgian laboratories.

Table 7 – Type of method used by laboratories in the ESP colon 2013 scheme

Lab characteristics	Number of labs	Number of Belgian labs
	KRAS	
Kit based tests	52 (39.7%)	9 (56.3%)
Home brew tests	48 (36.6%)	2 (12.5%)
NGS tests	12 (9.2%)	4 (25.0%)
Combination of methods	19 (14.5%)	1 (6.3%)
	NRAS	
Kit based tests	31 (29.8%)	3 (33.3%)
Home brew tests	51 (49.0%)	1 (11.1%)
NGS tests	11 (10.6%)	4 (44.4%)
Combination of methods	11 (10.6%)	1 (11.1%)

A part of the ESP EQA scheme is the evaluation of the clinical laboratory reports. Clear and concise written reports, readily interpretable by clinicians is essential for correct patient-management decisions. The written reports were evaluated for the presence and for the presence and correctness of different elements based on established standards such as ISO 15189. Evaluation of these reports revealed that a number of elements were well represented, namely patient identification, the results found and the applied methods. On the other hand, several essential elements were often missing, most notably a clinical interpretation, method sensitivity, and the use of a reference sequence. It was striking that in the 2013 scheme, several months after introduction of *NRAS* as a predictive marker for colorectal cancer, 18% of laboratories still made treatment recommendations based on an incomplete RAS test.(personal communication E. Dequeker)

As table 8 illustrates different methods, including NGS can be used to analyze the samples which are provided by the different EQA providers. As a pilot scheme EMQN organized last year a scheme specific for NGS and other high-throughput technologies with an oncogene panel. This panel includes mutations in the *EGFR, PIK3CA, KRAS, HRAS, NRAS, cKIT, TP53* and BRAF genes. This scheme was being offered to help labs accurately validate assay sensitivity and specificity (www.emqn.org). High quality reference materials are provided covering a range of genes with ddPCR quantified allelic frequencies. A remark should be made that sensitivity and specificity should be validated or at least verified by using real patient material as offered in diagnostic settings.

3.4 Quality assurance of NGS panel tests for somatic mutations

3.4.1 General considerations and the importance of quality

It is not within the scope nor the mandate of this report to establish guidelines or set method performance criteria for NGS testing. However, we wish to draw the attention to numerous challenges that NGS based assays encounter at the pre-analytical phase, the analytical phase and the post-analytical phase.

This test-cycle involves 3 major components: sample preparation, sequencing and data analyses. It is important to realize that various combinations of instruments, reagents and bioinformatic pipelines may be used for NGS based analyses and that standardization may be challenging. The complex nature of NGS based assays requires thorough assessment of potential pitfalls related to the different phases of the test-cycle. As any other laboratory test it is prone to sample contamination, sample mix-ups, tumour-normal switches. In addition procedures need to be established to cope with the inherent variability of the NGS experiment and to translate its outcome in a concise and correct report for the clinician. The setup of the NGS, including the software pipeline, is designed to reduce error to a minimum, eg false positives arising from sequencing errors that may be platform-specific (see table 9).

Table 8 – Examples of risk factors that may induce sequencing errors

Error prone sequencing risk factors for NGS	Reference
Homopolymeric sequences	Margulies et al., 2005[36]
Alignment artefacts	Koboldt et al., 2010[37]
Pseudogenes	Claes KB et al., 2014[38]
Different bioinformatics pipelines	O'Rawe et al., 2013[39]
High GC-rich regions	Bentley et al., 2008[40]

It is thus imperative that technical weaknesses of the chosen methodology are understood in order to assess these weaknesses during the validation of the assay.

The entire workflow determines the performance of NGS based assays. Validation of these assays should thus comprise all aspects of the test process, namely the sample preparation, the sequencing process and the data analysis. The used NGS platform needs to be validated for each type of variant (single nucleotide variants, insertions and deletions, and if part of the diagnostic assay also copy number variants, and other structural variations) that the test is designed to detect. The test validation has to demonstrate the ability to identify variants in the specific regions of the genome under investigation. The performance specifications of the data analysis pipeline should be established using appropriate reference materials that may include electronic reference data files that contain sequences that are simulated or based upon actual patient samples, or other reference materials, such as characterized gDNA from cell lines, but also real FFPE samples. The analytical performance specifications of the optimized assay and bioinformatic pipeline needs to be established in order to confirm that the test is suitable for its intended use.

There are several international guidelines and standards (see list below, including the overview by Bennett et al, 2014[41]) that can be used as a reference documents when implementing any NGS-technology for diagnostics. ISO 15189 emphasizes the quality of contributions to patient care, as well as that of laboratory (technical) and management procedures. Article 33bis, mentions as one of the requirements for reimbursements of tests an ISO15189-accreditation conform ISO 15189, verified by BELAC and is in other words the minimum for implementing NGS in routine daily practice in the clinic.

The following international and national NGS-related guidelines are available for consultation:

1. College of American Pathologists' Laboratory Standards for Next-Generation Sequencing Clinical tests.(2014)[42]
2. Royal College of Pathologists of Australasia (RCPA). Implementation of Massively Parallel Sequencing in Diagnostic Medical Genetic Testing. Available online: http://pathwiki.rcpaqap.com.au/pathwiki/index.php/Introduction (accessed December 2014).
3. New York State Department of Health. "Next Generation" Sequencing (NGS) guidelines for somatic genetic variant detection. Available online: http://www.wadsworth.org/labcert/TestApproval/forms/NextGenSeq_ONCO_Guidelines.pdf (accessed December 2014).
4. New York State Department of Health. Test Approval Policy. Available online: http://www.wadsworth.org/labcert/TestApproval/ (accessed December 2014).
5. US Centres for Disease Control and Prevention (CDC). Next-generation Sequencing: Standardization of Clinical Testing (Nex-StoCT) workgroup. (USA, 2012)[43]
6. U.S. Food and Drug Administration. Ultra High Throughput Sequencing for Clinical Diagnostic Applications—Approaches to Assess Analytical Validity, Silver Spring, MD, USA, 23 June 2011.
7. Weiss, M.M.; van der Zwaag, B.; Jongbloed, J.D.; Vogel, M.J.; Bruggenwirth, H.T.; Lekanne Deprez, R.H.; Mook, O.; Ruivenkamp, C.A.; van Slegtenhorst, M.A.; van den Wijngaard, A.; et al. Best practice guidelines for the use of next-generation sequencing applications in genome diagnostics: A national collaborative study of Dutch genome diagnostic laboratories. Hum. Mutat. 2013, 34, 1313–1321.
8. Schrijver, I.; Aziz, N.; Farkas, D.H.; Furtado, M.; Gonzalez, A.F.; Greiner, T.C.; Grody, W.W.; Hambuch, T.; Kalman, L.; Kant, J.A.; et al. Opportunities and challenges associated with clinical diagnostic genome sequencing: a report of the Association for Molecular Pathology. J. Mol. Diagn. 2012, 14, 525–540.

9. Rehm, H.L.; Bale, S.J.; Bayrak-Toydemir, P.; Berg, J.S.; Brown, K.K.; Deignan, J.L.; Friez, M.J.; Funke, B.H.; Hegde, M.R.; Lyon, E. ACMG clinical laboratory standards for next-generation sequencing. Genet. Med. 2013, 15, 733–747.[44]
10. Ellard, S.; Lindsay, H.; Camm, N.; Watson, C.; Abbs, S.; Mattocks, C.; Taylor, G.R.; Charlton, R. Practice guidelines for targeted Next Generation Sequencing analysis and interpretation. Association for Clinical Genetic Science (ACGS). Available online: http://www.acgs.uk.com/media/774807/bpg_for_targeted_next_generation_sequencing_may_2014_final.pdf (accessed December 2014).
11. Clinical and Laboratory Standards Institute. Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline—Second Edition. Available online: http://shopping.netsuite.com/s.nl/c.1253739/it.A/id.1787/.f (accessed December 2014).
12. Eurogentest prepared a draft consensus document 'Guidelines for diagnostic next gen sequencing

Whereas the ISO15189 standard is generic in the sense that it can be applied to any type of laboratory, the College of American Pathologists have issued guidelines that are applicable in a more practical manner to diagnostic laboratory testing in the oncological setting. They distinguish essentially 2 processes within the NGS-flow, namely the analytical wet bench process and the bioinformatical analyses of the data.

3.4.2 Quality assurance for the wet and dry lab part of NGS

The NGS flow can generally be categorized in a wet- and dry-bench part respectively.

The **wet-bench** comprises essentially the handling of the patient samples, the assessment by the pathologist of the tumour cell nuclei content when it concerns FFPE-or frozen samples, the extraction of the nucleic acids from either FFPE, blood of frozen sections. NGS may be performed on any DNA/RNA sample as long as the quality and quantity of the input material enables the purpose for which the assay is intended for. Starting from fresh frozen tissue samples instead of FFPE material could be an alternative to avoid fixation-related artefacts. However, pragmatic considerations (necessity of a separate sampling procedure, the necessity to have adequate storage capacity, the necessity to use archived FFPE-blocks, etc…) make it difficult to currently recommend fresh frozen tissues instead of FFPE-tissues as the matrix that should be used for diagnostic testing. This recommendation may change if in the future other analyses become clinically relevant that require fresh frozen tissue.

The importance of pre-PCR processing is illustrated by a recent EQA ring-study demonstrating that the rate of mutation detection failure across 13 molecular pathology laboratories for BRAF and EGFR-mutation testing on FFPE-samples was 11.9%, of which 80% of these were attributed to pre-PCR errors.[45]

When considering FFPE-tissues the loss in specificity of the assay may be related to DNA-fragmentation and non-reproducible sequencing artefacts after PCR amplification.[46] In a study of 488 FFPE samples sequencing artefacts were assessed in the 1-10% and 10-25% allele frequency range. These artefacts were inversely associated with coverage, indicating that more sequencing artefacts were associated with low amounts of available template, rendering a higher risk of false positives. This suggest that this type of assessment should be undertaken for all actionable variants when FFPE-blocks are concerned, which is the case in a routine diagnostic setting for solid tumours since frozen tissues are not that extensively available than FFPE-blocks. Rare false positives for actionable mutations have thus been found using NGS panel tests and this may be more likely to occur when the coverage and the number of reads are low and in case of bad quality DNA, as can be seen after fixation and storage.

In the sample preparation step a sequencing library is constructed for a specific sequencing platform. DNA fragments are generated with platform specific adaptors that often include molecular barcodes that enable sample pooling before sequencing.

As required by ISO15189- and the complementary NGS CAP-checklists, the full detail of this process in the lab should be validated and described in a validation report and standard operating procedures SOPS so that each step can be fully traced.

An example of the optimization of the NGS workflow is demonstrated by Choudhary et al.[47] In this study, the workflow was evaluated by sequencing mixtures of intact DNA to establish analytical sensitivity and precision, utilization of heuristics to identify systemic artefacts, titration studies of intact

DNA and FFPE samples for input optimisation, and incorporation of orthogonal sequencing strategies to increase both positive predictive value and variant detection. They also analyzed several FFPE-samples to assess clinical accuracy and incorporated a quantitative functional index (QFI) for sample qualification as part of the complete system performance.

Some practical examples to illustrate the details of information and validation that is expected:

- If target enrichment protocols are used, these should be fully documented with detailed information on the genomic regions captured.
- If samples with different matrices are used, for example blood vs frozen vs FFPE (formalin fixed and paraffin embedded), SOPs should be developed for each validated sample type.

As an example, when using FFPE-samples different levels of neoplastic content and different levels of cellularity representing a wide variety of tumour types should be included in the validation to:

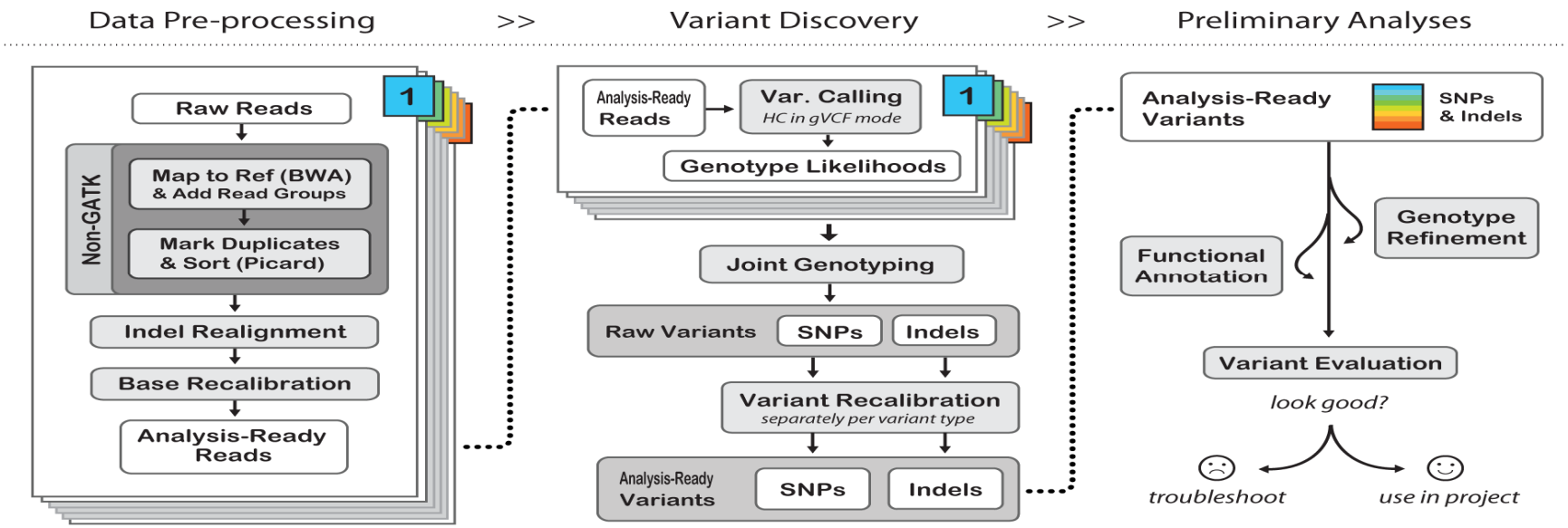
- model best the power for NGS experiments and appropriate read depth,
- determine the limit of detection of the platform,
- determine the uniformity of coverage,
- determine the depth of coverage required to confidently detection mutations within a tumour,
- all this in addition to classical parameters such as the accuracy, analytical sensitivity and analytical sensitivity.

The data analyses phase, or the **dry bench phase** of the test cycles starts after that the sequencing library or a pool of libraries is loaded on the sequencing platform and sequencing data are generated. The bioinformatics processes can be conceptualized into 3 major steps:

1. Data production: the generation of a sequence read file by instrument-specific software.
2. Processing and event detection: identifying variations compared to reference sequence (human reference genome)
3. Variant annotation and interpretation within the context of the patient's phenotype to render a clinical report.

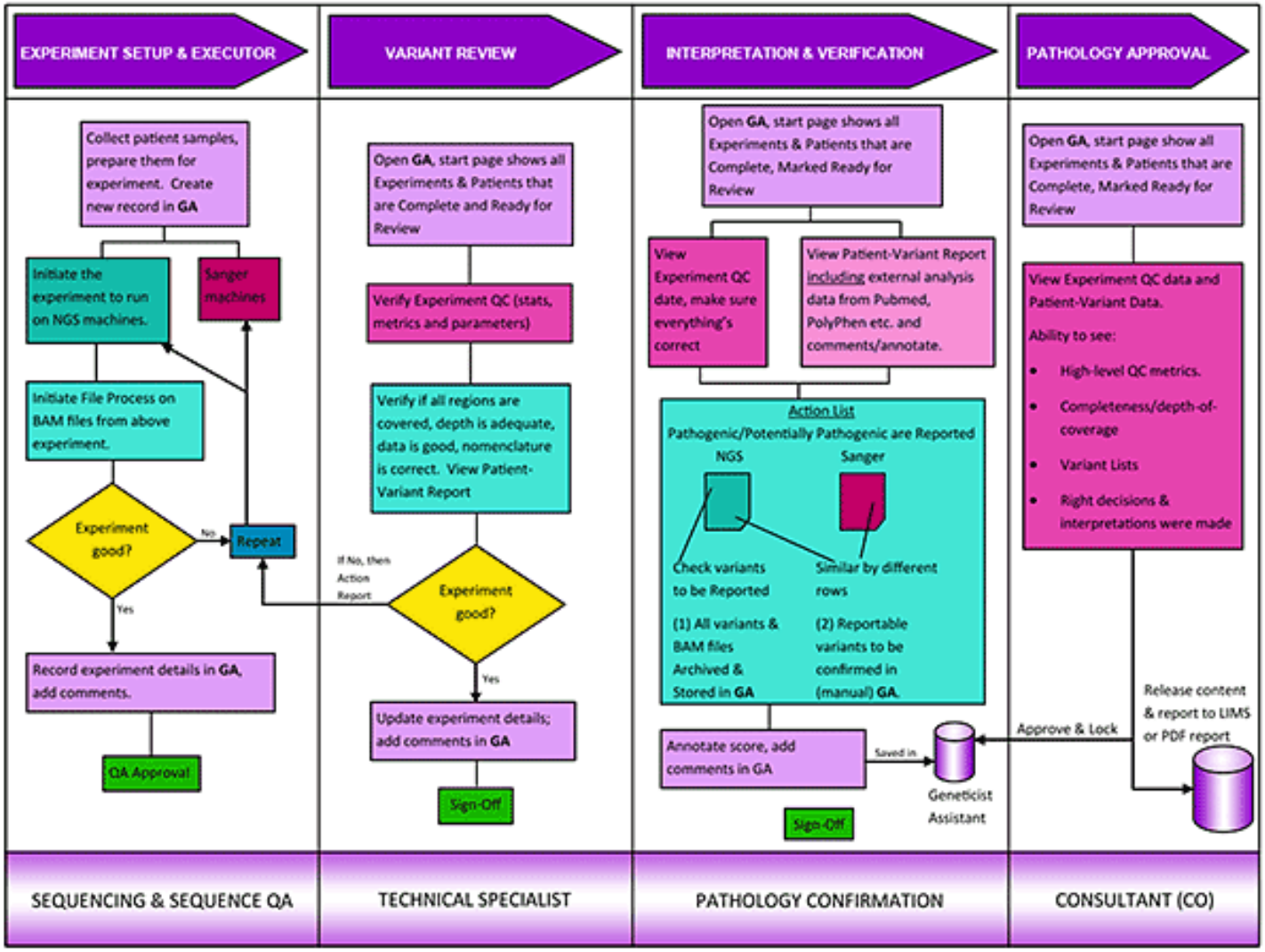
An example bioinformatic pipeline for variant calling, based on the Genome Analysis ToolKit[48, 49] is provided in Figure 4. The data processing and event detection part are depicted in the left most and middle panel. The Variant annotation and interpretation in depicted in the right most panel.

Figure 4 – Example of bioinformatics pipeline in a research setting that includes variant discovery, the Genome Analysis ToolKit[48, 49]



Source: Geraldine A. Van der Auwera, Mauricio O. Carneiro, Christopher Hartl, Ryan Poplin, Guillermo del Angel, Ami Levy-Moonshine, Tadeusz Jordan, Khalid Shakir, David Roazen, Joel Thibault, Eric Banks, Kiran V. Garimella, David Altshuler, Stacey Gabriel, Mark A. DePristo; From FastQ Data to High-Confidence Variant Calls: The Genome Analysis Toolkit Best Practices Pipeline; Curr. Protoc. Bioinform. 43:11.10.1-11.10.33. © 2013 by John Wiley & Sons, Inc.

Figure 5 – Example of bioinformatics pipeline in a clinical routine setting, the Geneticist Assistant NGS Interpretative Workbench



Source: Geneticist Assistant NGS Interpretative Workbench, http://www.mscience.com.au/view/genomics/soft-genetics (consulted February 5, 2015)

During data production the sequencing process generates a sequence read file consisting of a linear nucleotide sequence, with each nucleotide assigned a numerical value (termed its base quality/Phred score) that correlates to its predicted accuracy. This base calling process is typically integrated in the instrument software given the technology specific nature of the process. In case a pool of libraries was loaded, sequencing reads need to be sorted in different sequence read files based on the molecular barcodes (demultiplexing sequence data). Sequence read files are usually configured in the FASTQ file format, which contains the compilation of individual sequence reads, each with its own identifier, and an associated base quality score for each nucleotide. FASTQ files have become a dominant form of information exchange in the field of NGS.

The sample data are further processed for event detection. The sequence reads are aligned to a reference sequence (read-alignment) to identify differences between the obtained sequence reads and the reference (variant-calling). Identified variants may include single nucleotide variants, insertions and deletions, copy number variants, and other structural variations (translocations, inversions, etc). A wide variety of read-alignment methods and variant-calling algorithms are currently in use but comparative studies have shown a rather low concordance between variant-calling pipelines.[39] Stressing that, in a clinical setting, it is crucial that the implemented methodology is compliant to the most stringent quality assurance standards.

Finally, the detected variants need to be interpreted in the context of the patient's phenotype and a clinical report needs to be generated. This process includes the following steps: 1. Annotation and functional prediction; 2. Filtering, review and validation; 3. Interpretation and report generation to define ultimately its clinical application. First the Identified variants are annotated to provide information regarding their impact on gene and protein function. Annotation can be done with a variety of bioinformatics tools (commercial and non-commercial) and with different genomic resources (for example transcript set for ENSEMBLE and REFSEQ). The annotation step must therefore be considered carefully, and a conscious choice made as to which transcript set and software are used for annotation (McCarthy et al, Genomic Medicine). An observed variant can be annotated as clinically relevant based on different publically available resources or based on a local curated database of know/interpretable variants. As long as there are no consensus guidelines on the methodology to use, the laboratory needs to document fully the annotation pipeline that is being used, providing adequate reasoning on the resources used. Adequate personal training and an interpersonal concordance assessment needs to be performed in order to avoid interpersonal discordances when annotating the same variant. On the other hand, the laboratory could also predefine the clinically relevant, actionable vs potentially actionable variant and unknown, which is then being updated regularly when new knowledge arises. This policy needs to be documented appropriately. The option that is eventually chosen needs to be developed in collaboration with the end users of the data, namely the clinicians. Again the choice of resources will affect the annotation and interpretation of a known mutation. When it concerns a non-hotspot mutation, answering the question whether this mutation is biologically and/or clinically relevant is even more complicated. Several computational methods have been developed to discern which somatic mutation leads to which amino acid changes that could have biological consequences. Examples include for example PolyPhen, SIFT, etc… Also here it has become apparent that the mutation effect predictors varied significant in their accuracy, namely negative predictive value,[50] illustrating again that variant annotation is not trivial and difficult to implement in a clinical setting.

A non-limitative list of sources that can be used for variant annotation:

- PubMed: http://www.ncbi.nlm.nih.gov/pubmed/
- UniProtKB: http://www.uniprot.org/uniprot/
- COSMIC: http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/
- ClinVar: http://www.ncbi.nlm.nih.gov/clinvar/
- DGIdb: http://dgidb.genome.wustl.edu/
- Memorial Sloan Kettering cBioPortal http://www.cbioportal.org/
- ClinicalTrials.gov http://www.clinicaltrials.gov/
- MyCancerGenome: http://www.mycancergenome.org/
- Ensembl Variant Effect predictor: http://www.ensembl.org/Homo_sapiens/Tools/VEP

Once relevant mutations have been annotated and interpreted, they can be reported. An important, often overlooked, aspect of reporting on the results of an NGS experiment is the actual regions of the genome that were successfully assessed in the test. The laboratory needs to develop a policy on whether the findings would need additional validation, acknowledging the reasoning of the policy given. In other words, it is essential to report on the bases that the test covers were no mutation was observed or were not sufficient information was available for evaluating the state of some parts of the region of interest.

The samples used for validation will contain previously confirmed variants, as well as different types of mutations, for example mutations in homopolymeric regions, large indels, small indels since this the latter will demonstrate the performance of the platform, while finding the exact mutation demonstrate the performance of the assay used. or the identified variants may be confirmed post bioinformatics analysis. The validation may confirm that the bioinformatics tools and parameters are performing satisfactorily (eg, high specificity and sensitivity if the assay is a stand-alone assay for variant detection and reporting versus high sensitivity if it is a screening assay followed by a second assay that is used for confirmation) per laboratory requirements and clinical criteria for reporting, or adjustments or alternative tools may need to be further evaluated.

Once a satisfactory bioinformatics validation has been achieved, translation of the NGS assay into the clinical laboratory requires that the procedures are compliant with both the ISO15189 and CAP-standard.[42] In summary, the bioinformatics pipeline used to analyze, interpret and report NGS results needs to be documented in SOPs. A policy for dealing with pipeline updates and for the storage of the raw and analyzed data needs to be established. The version of the used pipeline is traceable for all generated NGS data files and deviations from the standard operating procedures need to be logged in an exception log. In case data transfer is part of the process it is required to establish a data transfer confidentiality policy for NGS data to ensure that Internal and external storage and transfer of sequencing data maintains confidentiality and security. The bioinformatic pipeline needs to be validated and revalidated each time modifications are made. The College of American Pathologists Guideline on NGS is therefore a welcome and imperative add to implement NGS pipelines within an ISO15189-accredited environment in a diagnostic setting.

3.5 Reporting of NGS data

Reports of NGS results should follow the general principles of clinical reporting and be in line with international diagnostic standards ISO 15189, and with professional guidelines. It is essential that results are reported in a clear and consistent manner, since laboratory reports may be read by both experts and non-experts. Eurogentest prepared a draft consensus document 'Guidelines for diagnostic next gen sequencing' (https://www.eshg.org/fileadmin/www.eshg.org/documents/EGT/EuroGentest-NGS-Guidelines-2014-FinalDraft.pdf). In summary this guideline suggest a one-page report with three supplements. It is essential to use the mutation nomenclature according to Human Genome Variation Society (HGVS; http://www.hgvs.org/mutnomen/) and to include genome build and reference sequence used for gene, transcript and variant description. The HGNC approved gene symbol should be used at least once, for reference. In addition, it is strongly recommended to include genomic coordinates in order to ensure uniform bioinformatics analysis and consistent documentation of identified variants. Exon annotation of the identified variants is not required since version updates of the reference sequences occur frequently. To fulfill administrative, clinical and technical requirements, a patient report should contain patient and sample identification, restatement of the clinical question, specification of genetic tests used, results, interpretation, and a final conclusion. The one-page report thus list all the essential data about the test. The rationale for offering a one page summary is that the clinician will probably only scan the summary, and not look at all the information. Hence, the clinically significant conclusions and the relevant test and test quality data should feature on the first page. The full report has to be much more elaborate, and contain much more details. We propose to work with supplements (or annexes) appended to the summary report, in which important test characteristics and details are described in addition to brief, clinical diagnostic report. Each page supplement should of course carry the patient identifier, a page number and the date, and unequivocally linked to the corresponding report.

One supplement is dedicated to test characteristics and bio-informatics details of targeted capture or exome sequencing. Exome sequencing in diagnostics is often initially restricted to the analysis of a disease-associated set of genes based on the patient's clinical indications. Therefore, it is

required to include a complete gene list which is diagnostically targeted in the capture assay as well as in the exome. This gene list should be selected by a team of experts and the validation of the assay should warrant that the listed genes are tested at high quality. Furthermore, a succinct but complete description of technical issues like the target enrichment approach, the NGS platform, and the data analysis pipeline used are required in the report. Versioning is very important in this respect, and a requisite of the report.

NGS testing meets new or other limitations in its performance and analysis compared with Sanger sequencing. It is therefore essential to include in the report disclaimers related to the test performance and the analytical limitations.

A second supplement would be specific for each patient and include some quality issues such as the quality of the tissue and other quality metrics specifically related to the performance of the assay. It is essential to report (analytical) performance related to the minimum threshold that is guaranteed for the test. It is strongly recommended to report the performance related to the clinical target which is used for analysis in a given sample. The minimum threshold, sensitivity and specificity should be evidence based and must have been established during the test validation process in accordance with the matrix used and the tumor cell content. It is recommended to include the total number of variants observed in the analyzed gene panel in this specific sample; this can be used as a monitoring quality parameter of the whole pipeline. In addition, it is required that the report states whether some regions were not well covered and not complemented by another technique in a given sample. Laboratories must be able to show detailed information about the regions that were not successfully sequenced or analyzed.

A third supplement would include the variants retained after analysis of the processed data in a clear and adequately structured format. The list of variants need to be narrowed down to a list of potential reportable candidate variants,[12] the variants in general can then be grouped in three categories:

1. Those that have direct patient impact, the so called “actionable” variants,
2. those that are biologically relevant, thus pathogenic, but not actionable and
3. those that have unknown significance.

When it comes to the interpretation of predictive variants it is emphasized that four different types of information should be used, namely

1. The gene of interest,
2. The specific variant within this gene,
3. The drug or class-of-agent sensitivity/resistance pattern and
4. The tumour type context.
5. When summarizing the variant findings, it is recommended to include the gene name, zygosity, cDNA nomenclature, protein nomenclature, genomic position. In addition to the report format, 5 other statements were made related to NGS reporting in the draft Eurogentest guideline (version October 2014).
6. The report of an NGS assay should summarize the patient’s identification and diagnosis, a brief description of the test, a summary of results, and the major findings on one page.
7. A local policy, in line with international recommendations, for reporting genomic variants should be established and documented by the laboratory prior to providing analysis of this type.
8. Data on unclassified variants’ or variants of unknown significance has to be collected, with the aim to eventually classify these variants definitively.
9. Laboratories should have a clearly defined protocol for addressing unsolicited and secondary findings, prior to launching the test.
10. The laboratory is not expected to re-analyse old data systematically and report novel findings, not even when the core disease genes panel changes.

To be able to manage disease variants, the laboratory has to set up a local variant database for the different diseases for which testing is offered on a clinical basis.

Currently, existing guidelines on reporting are related mostly to constitutional disorders. The Association for Clinical Genetic Science (ACGS) issued “Practice Guidelines for targeted next generation sequencing analysis and interpretation” (Ellard et al., 2014, see above) and the American College of Medical Genetics and genomics has also issued ACMG clinical laboratory standards for next-generation sequencing.[44] To what extent these

guidelines on the reporting of incidental variants are appropriate to cancer genomic testing is unclear when the test is ordered outside the clinical scope of detecting germline variants that convey cancer susceptibility. It is emphasized that the testing for oncology patients will be centered mostly around finding variants that might guide optimal therapy with respect to the genetic aberration found.[51] Nevertheless, especially with large panels, the handling of accidental germline findings might need to be defined appropriately, whenever considered applicable. This includes guidance on the need for pre-test counselling and discussed above.

Based on the existing guidelines related to reporting the following tables were generated to illustrate which elements could be included in the report. Based on several guidelines.

Table 9 – Reporting, elements to be included in the summary report (one page)

Eurogentest: NGS reporting guidelines	Gulley et al (2007). Clinical Laboratory Reports in Molecular Pathology. [52]	CAP (2012). Molecular pathology checklist. (04.21.2014)	ISO 15189:2012
Patient (each page)/sample identifiers	Patient/sample/laboratory identifiers		Patient identification on each page
	Patient's name, DOB, date of specimen collection, time of specimen collection, date of receipt or accession in lab (+ accession number), specimen source, how is the tissue received; accession number and specimen number from referring lab, ordering physician, Name of testing laboratory when transmitting a referral laboratory's results, name and address of reporting lab		Date of specimen collection, time of specimen collection, specimen source, ordering physician, Identification of reporting laboratory, identification of the person(s) reviewing the results and authorizing the release of the report
Restatement of the clinical question	Indications for testing (optional)		
Diagnosis	Clinical history (optional)		
Brief description of the test data and test quality data	List results by test name	Summary of the methods, mutations tested	
Summary of the results		Results	
Major findings/clinically significant conclusions	Analytical and clinical interpretation (optional)	Analytical and clinical interpretation	Interpretation
Date of report (each page)	Date of report		Date of report
	Signature of reporting physician and lab director if interpretation was done, identity of person who approves report	Signature of lab director if interpretation was done	
	Clinic/inpatient location or details of outside facility (optional)		Patient location (each page)

Table 10 – Reporting, supplement 1: test characteristics and bio-informatics details

Eurogentest: NGS reporting guidelines	Gulley et al (2007). Clinical Laboratory Reports in Molecular Pathology. [52]	CAP (2012). Molecular pathology checklist. (04.21.2014)	ISO 15189:2012
Gene list	Defined target	Loci/mutation	
Description of technical issues		Methods/ instruments/ reagents/ bioinformatics process (+ versions)	Identification of the examination including, where appropriate, the examination procedure (including those from referral laboratory)
The target enrichment approach, the NGS platform, and the data analysis pipeline used (version!)	Type of procedure/method (analyte-specific reagent or kit version and manufacturer, instrument type)		
Test performance and analytical limitations (the lack of coverage; the missing of a variant type; possibility of incorrect template mapping due to pseudogenes).	Information that may affect test interpretation	Limitations of the findings	Cautionary or explanatory notes
How the NGS test differs from previous tests	Correlation with prior test results (optional)		
Confirmation by another independent method?	Control test result if unusual of especially pertinent (optional)		
	Reference range		Biological reference intervals, clinical decision values
	Disclaimer on non-FDA-approved tests in which a commercial analyte-specific reagent was used (optional)	Disclaimer on non-FDA-approved tests in which a commercial analyte-specific reagent was used	

Table 11 – Reporting, supplement 2: quality issues and test performance data (patient specific)

Eurogentest: NGS reporting guidelines	Gulley et al (2007). Clinical Laboratory Reports in Molecular Pathology. [52]	CAP (2012). Molecular pathology checklist. (04.21.2014)	ISO 15189:2012
(Analytical) performance: the minimum threshold that is guaranteed for the test	Assay performance characteristics; significance of the results		
Total number of variants observed in the analyzed gene panel in this specific sample			
Regions that are not enough covered and not complemented by another technique in a given sample			
Regions that were not successfully sequenced or analysed			
Gaps filled by Sanger (or other means) that are not attainable using NGS			
Diagnostic yield of the test, if possible.			
	Condition of the specimen (optional); reason specimen was rejected (optional)	Condition of the specimen; reason specimen was rejected	When the quality of the primary sample received is unsuitable for examination, or could have compromised the result, this is indicated in the report.

Table 12 – Reporting, supplement 3: variants retained after analysis

Eurogentest: NGS reporting guidelines	Gulley et al (2007). Clinical Laboratory Reports in Molecular Pathology. [52]	CAP (2012). Molecular pathology checklist. (04.21.2014)	ISO 15189:2012
gene name, zygosity, cDNA nomenclature, protein nomenclature, genomic position	Use standardized gene nomenclature and standard units of measure	Use standardized gene nomenclature and standard units of measure	

3.6 Needs for education and a multidisciplinary expert committees

Many health professionals need to be trained in genomic methods to keep pace with the rapid developments in this area. The level of knowledge required will differ depending on the role of the health professional in the chain of custody of the molecular analysis pipeline. The ultimate goal is to make a contextual interpretation of the data, based on the clinical question and finally effectively report the results to the clinician.[53] Expertise should be available for the following areas: 1. To understand the NGS approaches in genomic pathology (for example hybrid capture vs amplification based methods, the number of genes used, the different classes of mutation that can be detected, depth of coverage that can differ between amplification and -capture based approaches for targeted sequencing), 2. Assay design and validation, 3. Bioinformatics and 4. Reporting.

It is increasingly recognized that pathologists/clinical biologists/geneticists are gatekeepers to ensure appropriate test utilization and cost-effective ordering patterns. At a large reference laboratory, approximately 30% of the requests for 36 molecular tests were inappropriate. In many of these cases the test was wrongly ordered (http://www.aruplab.com/files/resources/genetics/White-paper-1-value-of-GCs-in-lab.pdf).

In addition, a study on 26 laboratories and with 2240 test requests was performed by the College of American Pathologists Quality Practice Committee (reported November 2014) where the following variables evaluated: 1. the appropriateness for molecular testing; 2. the adequacy of test material and 3. the turnaround time. In brief, when it concerns the appropriateness of molecular testing, the results show suboptimal compliance with international guidelines such as the National Comprehensive Cancer Network (NCCN) when it concerns practicing molecular testing. One of the main reasons of non-compliance is not necessarily that someone is not willing to adhere to a guideline, but because there is large variability on how to use existing guidelines that all may not be coherent between each other, illustrating the need to develop a national genomic molecular markers dataset that should contain the minimum genes to be tested uniformly across all Belgian laboratories. (http://www.captodayonline.com/molecular-ap-testing-sync-guidelines/, consulted December 2014)

In general though, physicians are aware of their lack of genomic knowledge. In a 2013 study of 200 internist, 74% rated their knowledge as “somewhat poor” or “ very poor” and approximately 80% of them indicated a need for additional training.[54] Therefore, there may be a need to implement educational activities at all levels of clinical practice, starting from the residency program of the different specialties (pathology, clinical biology) to the practising specialist.[55] It is emphasised that not only pathologists are important in this genomic area setting, but also geneticists and clinical biologists.

Genomics-related patient care necessitates a multidisciplinary approach, with the instalment of “molecular advisory boards” or “molecular sequencing boards” that includes expert clinicians, molecular pathologists or clinical biologists, and geneticists whenever it concerns solid tumour and/or haematological testing, scientist, ethicist whenever applicable and bio-informaticians.

There are many challenges for interpreting genomic data in cancer by molecular advisory boards as illustrated in a publication by Dienstmann et al., 2014:[56]

“Is this an activating or inactivating mutation?

- Some mutations are activating and confer oncogene-addiction in specific contexts (*FGFR2*^{S252W} or *FGFR2*^{N549K} in endometrial cancer, effectively targeted by FGFR inhibitors ponatinib and BGJ398 in preclinical models). Others generate markedly reduced kinase activity and loss-of-function (*FGFR2*^{R251Q} and *FGFR2*^{I648T} in melanoma, with no predicted benefit with FGFR targeting).(Gartside et al., 2009; Gozgit et al., 2012 and Guagnano et al., 2012)

Does this mutation engender sensitivity to specific targeted therapeutics?

- Some activating mutations based on *in vitro* models do not confer sensitivity to agents targeting the mutant protein (*AKT*^{E17K} in breast cancer, effectively targeted by non-allosteric AKT inhibitors but not by allosteric AKT inhibitors; *MEK*^{C121S} in melanoma, not inhibited by allosteric MEK inhibitors).(Carpten et al., 2007, Jo et al., 2011 and Wagle et al., 2011)
- With inhibitors of the mutant kinase, level of sensitivity depends on the potency of the agent (*EGFR*^{T790M} in lung cancer, resistant to first-generation EGFR inhibitors but higher sensitivity to novel irreversible inhibitors;*ABL1*^{T315I} in chronic myeloid leukemia, resistant to imatinib and sensitive to ponatinib; *ERBB2*^{L755S} in breast cancer, resistant to lapatinib and sensitive to neratinib).,(Bose et al., 2013, Cortes et al., 2012 and Sequist et al., 2013)
- With downstream pathway inhibitors, level of sensitivity strongly depends on the functional effects of the mutation (activating *BRAF*^{L597} and *NRAS*^{Q61}mutations confer sensitivity to MEK inhibitors in melanoma; *BRAF*^{Y472C} reduces kinase activity and confers sensitivity to dasatinib in lung cancer, but not to MEK inhibitors).(Ascierto et al., 2013, Dahlman et al., 2012 and Sen et al., 2012)
- For rare variants in oncogenes, there is no definitive preclinical or clinical data to suggest sensitivity or resistance to targeted therapy (activating *PDGFR* mutations in the tyrosine kinase domain render tumours susceptible to PDGFR inhibitors in gastrointestinal stromal tumours, but the sensitivity of novel variants in the transmembrane domain of the gene is currently unknown).(Heinrich et al., 2003)
- Mutations that predict responsiveness to a therapy in some contexts, such as BRAF inhibitors in *BRAF*^{V600E} mutant melanoma, may be associated with entirely different clinical interpretations in others. In colorectal cancer, for example, *BRAF*^{V600E} mutations would direct towards combination of targeted therapies (BRAF plus EGFR inhibitors). It is important to emphasize that sensitivity is tumour context-specific and is influenced by concomitant genomic alterations.(Prahallad et al., 2012)

How to select therapy in case of multiple genomic alterations?

- The finding of concomitant genomic alterations in a tumour sample collected before systemic therapy can have important biological and therapeutic implications (in the setting of *BRAF*^{V600E} mutant melanoma, *NF1* loss predicts resistance to single-agent BRAF inhibitors; identification of *BRAF*^{V600E}and *MEK*^{P124S} in melanoma does not predict resistance to BRAF inhibitors; in *KRAS* mutant lung cancer, loss-of-function *STK11* mutations engender resistance to the combination of MEK inhibitors and docetaxel).).(Chen et al., 2012, Shi et al., 2012, Wagle et al., 2011 and Whittaker et al., 2013)
- In relapsed samples after targeted therapies, identification of “acquired” genomic alterations may be linked to resistance mechanisms and help define subsequent therapies (in *EGFR*-mutant lung cancer progressing to erlotinib, the finding of *MET* amplification directs to combination therapy with EGFR and MET blockade.(Engelman et al., 2007)
- On the other hand, prioritizing therapy in the setting of multiple “targetable” alterations is not as straightforward as in previous examples, especially when both targeted drugs are still in early phases of clinical development (activating K*RAS*^{G12D} mutation in pancreatic cancer, targeted by downstream PI3K pathway + MEK inhibitors, often coexist with *CDKN2A* loss-of-function mutations, which may theoretically predict sensitivity to CDK inhibitors; in prostate cancer, *PTEN* deletion directs to PI3K pathway inhibitors and the coexistence of *BRCA2* loss supports the use of PARP inhibitors. (Carver et al., 2011, Fong et al., 2009, Hofmann et al., 2012 and Stone et al., 2012)”[56]

Furthermore, in contrast to current NGS practice (often using a 4-5% detection cut-off), the detection of even lower proportions of altered DNA (e.g. 1%) may also have clinical management implications, stretching the NGS technical limits and increasing costs. For example, patients with advanced colorectal cancer with a low frequency (1% to 10%) KRAS mutation did not respond to anti-EGFR treatment,[57, 58] indicating low frequency KRAS mutations should be assessed.

There are several international initiatives ongoing that aim to determine to develop NGS-standards for clinical utility that seek to strike a balance between the development of quality evidence for decision-making and rapid assimilation of genomic information for patient care in a health care setting. A possible option could be to install a National NGS-Oncology Committee that is (preferentially) embedded in an international consortium that regularly updates the clinically relevant markers in oncology and interacts with the National Federal Authorities and reimbursement institutions. This consortium could be based on the Belgian model for hereditary genetic testing and could preferentially include experts from all disciplines involved, namely expert molecular pathologists, expert clinicians, bio-informaticians, and geneticists whenever applicable. An illustrative example that this board may tackle is the following: in one study exomes were sequenced in 32 patients with head and neck cancer identifying 17 missense mutations in Notch1 in 21 individuals,[59] whereas a second study sequencing samples from the same tumour type identified in 92 patients 7 Notch1 missense mutations in 11% of studied cancers.[60] Between these studies there was no overlap between the 2 sets of missense mutations, which may be a legitimate outcome of course, but this indicates the need for an assessment of reproducibility of the technical aspects of these studies. In a similar reasoning, this is national board could evaluate the reasons of discrepancy between different centres in a ring EQA-study.

Key Points

- **External quality assessment for molecular tests in Belgium needs to be streamlined**
- **The existing EQA results show there is room for quality improvement**
- **Guidelines are available to improve quality of NGS panel tests but no generally accepted minimum criteria exist for many aspects**
- **In the context of accreditation of NGS the NGS CAP guidance document should preferably be used together with ISO 15 189.**

4 IMPACT OF TEST ACCURACY ON INCREMENTAL COST-EFFECTIVENESS RATIOS

4.1 Introduction and scope

This chapter provides an overview of studies evaluating the cost-effectiveness of the combination of molecular diagnostics and targeted therapies in confirmed neoplasms. More specifically, it studies the impact that changes in test accuracy (i.e. diagnostic sensitivity and specificity) may have on the economic value of test-intervention combinations. The impact on the cost-effectiveness of the targeted therapy of using less accurate tests in clinical routine as compared with the centralised tests used in confirmatory RCTs is illustrated by means of specific examples.

4.2 Methodology

4.2.1 Search strategy

A systematic search for relevant publications was carried out with the consultation of electronic reference databases up to the first of August 2014. PubMed, EMBASE, Econlit (through OVID), NHSEED (CRD) and NHSHTA (CRD) were searched to retrieve primary full economic evaluations (studies comparing both costs and outcomes) and reviews of economic evaluations (i.e. secondary economic evaluations), which looked as part of their analysis at the impact of test accuracy on their overall results. Systematic reviews of the economic literature were used for checking purposes, in order to ensure no primary economic evaluations had been missed from our review.

An overview of the search strategy is given in Appendix 3. Furthermore, a communication to all (HTA) institutes listed on the INAHTA website (International Network of Agencies for Health Technology Assessment) was sent and the EunetHTA Pop database consulted, in order to identify any ongoing economic evaluations on the combination of molecular tests and interventions. No restrictions were imposed for language. Time of publication was limited to studies published on 2005 or later, given the relative novelty of the topic. Details of the search are available in the Appendix 3.

4.2.2 Selection procedure

To identify potentially relevant studies for our analysis we first went through all titles and abstracts in order to exclude any obvious studies that did not match our research subject. All articles that appeared to be interesting, or for which there were some doubts, were read in full in order to select those relevant for inclusion in our review. Reference lists of the selected primary and secondary economic evaluations found via our search were checked for additional references worth adding to our analysis. Study selection was completed by one researcher (LS) but any doubts that came up during the exercise were discussed and solved in collaboration with a second reviewer (FH).

4.2.3 Selection criteria

All full economic evaluations looking at molecular testing in confirmed neoplasms treatment decisions were considered for inclusion in this review. Cost descriptive analyses or cost comparisons not taking into consideration effectiveness were discarded. Similarly publications in the form of letters, editorials or notes and abstracts were excluded, since these would not offer enough information to include them in our analysis and critically appraise their findings.

Our search returned 462 citations, after eliminating duplicates. Of those, 408 did not meet our inclusion criteria based on a review of their title and/or abstract. Of the 54 citations left, 45 were excluded after reading their full text mainly because of the study design (no full economic evaluation), the indication (not in confirmed neoplasms) or the lack of analysis of the potential impact that changes in test accuracy could have on the cost effectiveness of the test-intervention combination, which left us with nine relevant studies to be included in our review. Further exploration of the references of the selected articles did result in two additional references, which resulted in 11 relevant economic evaluations. Although overall, our search identified very few studies looking at test accuracy and its impact on overall cost effectiveness, the following section offers a short description of those studies which covered our area of interest.

4.3 Results

Eleven studies overall looked at variations in test accuracy (sensitivity and/or specificity) as part of their sensitivity analyses, with three of them reporting very limited impact[61-64]. The remaining of the evaluations did find test accuracy to be a factor that could modify the overall base case results reported.

Retel et al. (2010) published a study on the cost-effectiveness of the 70-gene signature compared to St. Gallen guidelines and Adjuvant Online for treatment decisions in early breast cancer patients[65]. Although their base case scenario showed the 70-gene signature to have the highest probability of being cost-effective at a willingness to pay above €4 600, their sensitivity analysis, showed that varying the sources for the specificity and sensitivity data resulted in Adjuvant online! being a more effective and less costly option. An additional cost-utility study in the same area published by Oestreicher, et al. in 2005[66] compared gene expression profiling against NIH guidelines in breast cancer patients. On the one hand, the authors reported that although the gene profiling test under evaluation identified 35% fewer women to be treated with chemotherapy, gains in quality of life (QoL) resulting from reducing chemotherapy treatment were outweighed by the decrease in life expectancy due to the gene profiling test's lower sensitivity. On the other hand, the specificity of the test under study was 10-fold higher than that of the NIH guidelines, leading to lower overall costs. They added that if the sensitivity of the gene expression profiling test were to increase to at least 95% and its specificity was maintained (at 51%), it would improve QoL by allowing some women to safely avoid chemotherapy while not missing women whose survival could be compromised by such avoidance.

The example of trastuzumab was explored in three studies. Dendukuri et al.[67] estimated the incremental cost per accurate diagnosis for different testing strategies (FISH versus IHC). The evaluations concluded that the most cost effective diagnostic alternative was to test all newly diagnosed patients with IHC and then confirm scores of 2+ or 3+ by means of a FISH test.

In their sensitivity analysis the levels of test accuracy were explored. The authors mentioned that the rate of false positives, specifically for those with a IHC score of 3+ could be as high as 7.6%, which would translate into an important number of women being exposed to the risks of trastuzumab with no chance of benefiting from the treatment for their base case scenario (IHC followed by treatment of all 3+ and confirmation by means of a FISH test of 2+, then treatment of those 2+ confirmed). If the HER2 status of those women was accurately determined (0% false positives) by testing all patients with IHC and then using FISH to confirm all 2+ and 3+ results , the cost of trastuzumab would be reduced by approximately $0.6 million per 1000 women screened per year.

Two further studies by the same author confirmed that increasing the accuracy of IHC testing decreased the cost-effectiveness of FISH testing alone, making it still an attractive option for confirming certain IHC scores.[68, 69].

Two studies looked at testing for anaplastic lymphoma kinase (ALK) to guide decisions on crizotinib use in non-small cell lung cancer. One of these found that small decreases in the specificity of the test (1%) had a noticeable impact on the mean test cost per patient (€205), making the test more costly and less attractive from an economic perspective.[70], while the other[61] noticed a small decrease in the baseline ICER when modifying the specificity of the IHC test.

One study by Lee et al. (2013), focusing fully on the importance of test accuracy was identified.[71] The authors describe a simple approach to measure costs and effectiveness of companion diagnostics, taking into consideration important factors such as the prevalence of the biomarker or the accuracy of the test. They then move on to applying their approach to two different examples: use of trastuzumab in HER2 + breast cancer patients and KRAS testing in colorectal cancer.

Overall, the authors show via their examples that test accuracy is, together with the incremental cost of the targeted therapy and the incremental gains it offers, a crucial factor that could have a significant impact on the overall results. The weight of test accuracy appears to be superior to the influence that test prices have in the overall results, even when prices are considered high.

In addition to the primary economic evaluations identified via our search and described above, a recent systematic review published after the completion of our literature search, looked at the methodological challenges in economic modelling for companion diagnostics.[72] Their results highlight the importance from a methodological perspective of including test accuracy versus purely focusing on the cost of the test. It also discusses the potential weight that the prevalence of the biomarker and the timing/sequence of multiple testing could have on the overall model results.

4.4 Illustrative examples for Belgium

In order to make the examples more relevant for the Belgian situation the overall approach used by Lee et al.[71] was used and populated with Belgian data from a cost-effectiveness analysis undertaken from a payer perspective by the Belgian Healthcare Knowledge Centre in 2006 on trastuzumab (KCE report 34).[73] The study compared treatment with trastuzumab versus no trastuzumab over a lifetime period.

4.4.1 Data sources

Published trastuzumab trials in early stage breast cancer and, in particular for our example, data from the HERA, B31/N9831 trial was used to extract clinical data. Incremental health gains were measured in life years gained (LYG). Costs included charges for diagnostic, treatment or follow-up procedures associated with breast cancer management itself or with adverse reactions to trastuzumab treatment. Costs both covered by the RIZIV/INAMI and by the patient were taken into consideration.

The model looked at the gold standard, the FISH test (accuracy of the FISH test assumed to be 100%), and compared it to:

1. immunohistochemistry (IHC) test, followed by treatment of both 2+ and 3 + or only 3 + patients
2. or IHC test, followed by confirmation of 2+ and 3+ via FISH and treatment of all confirmed.

The sensitivity and specificity for the IHC options and the prevalence of the biomarker were taken directly from the Belgian report previously mentioned. Similarly the incremental cost of therapy and the incremental gains, measured as life years gained (LYG) were also extracted from the same source.

The potential effect of variations in accuracy levels can be easily explained on theoretical grounds; on the one hand higher sensitivity rates mean that a higher number of patients likely to benefit from the targeted therapy can be identified and consequently higher life years gained (LYG) can be realised. On the other hand, higher specificity rates help to reduce the potential treatment of "false positives" and consequently engaging in high spending for a proportion of the patient population for which the targeted therapy would not be effective.

4.4.2 Results and discussion

With this in mind, Table 14 gives an overview of the overall findings. While the gold standard (100% sensitivity and specificity) shows an ICER of €15 344/LYG, when the sensitivity is reduced to 67,0% and the specificity to 97%, as in the case of IHC testing and treating 3+, the overall ICER increases to €18 619/LYG. A scenario with higher sensitivity (96,2%) but lower specificity (88,0%), testing by means of IHC and treating 2+ and 3+ scores, showed an even higher ICER. This is primarily due to the lower specificity of such testing strategy which implies treating an important number of false positives, without offering any gains in terms of clinical benefits. The current situation in Belgium consists on IHC testing followed by FISH testing only for those 2+ and 3+ (23% of total), in order to confirm positive cases, after which treatment with trastuzumab is pursued for all confirmed. This "Belgian" strategy displays an ICER versus no treatment with trastuzumab slightly lower than the one seen for the gold standard with both the overall incremental cost and the incremental benefits linked to the Belgian approach slightly lower than those of the gold standard.

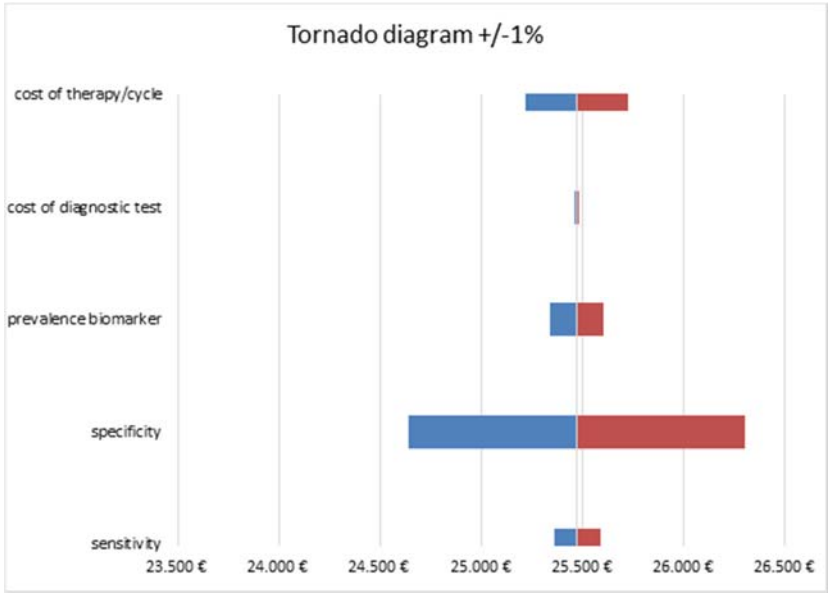
It is important to highlight that this is the case even if the specific cost of the test is higher for the "gold standard" (assumed as €341 based on reimbursement rates) than for the IHC scenarios (€78). This is because, as already mentioned by Lee et al. in their original article[71], in this type of test-intervention evaluations, the overall cost of the test would need to be very high to become an important factor, while the accuracy of the test results remain a more crucial factor. Despite the fact that test accuracy has more impact on the overall ICER than the test cost, the cost of the test has been studied and discussed more frequently in any of the evaluations performed up to date for test-therapy combinations.

Table 13 – Cost effectiveness of different test alternatives for targeting treatment with trastuzumab in breast cancer

# of newly diagnosed BC in BE/year	10.490	Scenario 1	Scenario 2	Scenario 3	
Variable	**Gold Standard - FISH test**	**IHC - treat IHC 3+**	**IHC - treat IHC 2+ & 3+**	**IHC plus FISH for 2+ and 3+ only**	**Source used**
Sensitivity of test	100,0%	67,0%	96,2%	96,2%	KCE report 34, 2006
Specificity of test	100,0%	97,0%	88,3%	100,0%	KCE report 34, 2006
Prevalence of biomarker	13,5%	13,5%	13,5%	13,5%	KCE report 34, 2006
True +	13,5%	9,0%	13,0%	13,0%	
False +	0,0%	2,6%	10,1%	0,0%	
Total +	13,5%	11,6%	23,1%	13,0%	
Cost of diagnostic test	€ 341	€ 78	€ 78	€ 158 (incl. FISH in 23%)	Reimbursement rates (2014)
Incremental cost of therapy	€ 29 852	€ 29 852	€ 29 852	€ 29 852	HERA results for stage II (KCE report 34, 2006).
Cycles of therapy for HER2 true + patient	18	18	18	18	KCE report 34, 2006
Cycles of therapy for biomarker false + patient (assumed)	18	18	18	18	Assumed equal to those for true +
Total incremental costs	€ 45 848 935	€ 37 248 420	€ 73 246 940	€ 42 344 066	
Incremental benefit of biomarker true + patient (LYG)	2,11	2,11	2,11	2,11	HERA results for stage II (KCE report 34, 2006)
Incremental benefit of biomarker false + patient (LYG)1	0	0	0	0	
Total incremental benefit (LYG)	2988,08	2000,52	2875,43	2875,43	
Share of Eligible Population Tested	100,0%	100,0%	100,0%	100,0%	Assumed
ICER (vs no treatment)	€ 15 344	€ 18.619	€ 25 473	€ 14 726	

[1] *No detrimental effect assumed for treating false positives, although in practice this could bring in losses (e.g. in terms of QoL) which would make the specificity of a test more crucial*

Figure 6 – Impact of a 1% change of input variables on the overall ICER of trastuzumab in HER2+ early stage breast cancer



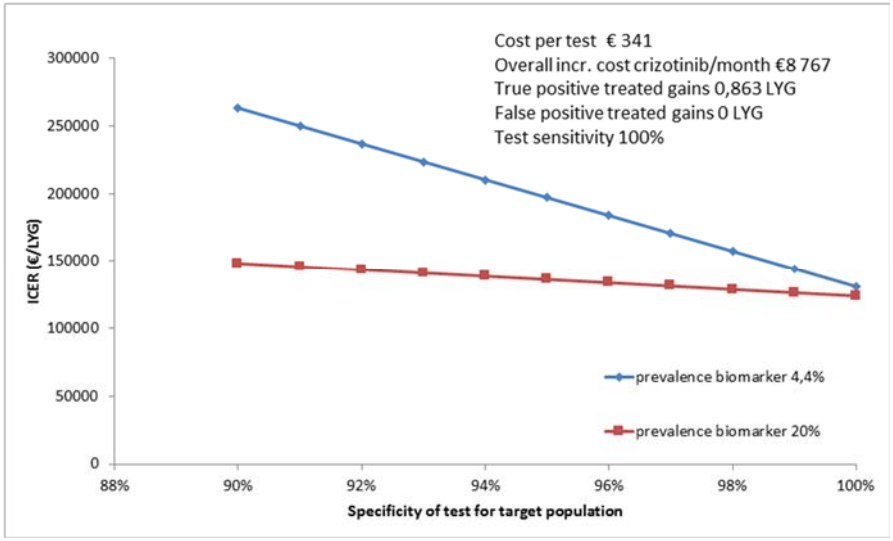
The importance of accuracy on the overall economic value of a test become even more crucial in those cases in which the prevalence of the biomarker is small, since under such scenario, small variations on the specificity of a test can have an important impact on ICERs. In order to illustrate this, the previously shown estimation have been applied to ALK testing in crizotinib treatment decisions for non-small lung cancer. The prevalence of the biomarker is in this case in the order of 4,4%. Input data used for our calculations are displayed in Table 15. Data on (discounted) incremental costs and benefits are taken from the Belgian evaluation of Xalkori (Dossier 4677.1, available from RIZIV/INAMI upon request) dating from 2012. The comparator used was docetaxel and the overall incremental costs and benefits were calculated over a 6-year time horizon.

Costs considered include drug acquisition costs, administration and monitoring costs, costs of managing adverse events and costs of progression and palliative care. For the purpose of our exercise the sensitivity of the test is held constant at 100%, while the specificity is gradually lowered by 1% from 100% to 90% and the hypothetical ICERs are calculated. At a prevalence for the biomarker of 4,4, variations in the ICER caused by reductions in the specificity of the test are noticeable (from €130 873/LYG for a test with a specificity of 100% to €263 302/LYG for a specificity of 90%). Figure 5 illustrates these variations together with those that would take place if the prevalence of the biomarker was 20% instead of 4,4%, keeping all other variables constant. As the graphic shows, the influence of test accuracy on the ICER diminishes greatly as the prevalence of the biomarker increases.

Table 14 – Cost effectiveness of different test alternatives for targeting treatment with crizotinib in NSCLC

ALK for crizotinib therapy - Lung		
Variable	**Gold standard test**	**Source used**
Sensitivity of test	100,0%	Assumed
Specificity of test	100-90%	Assumed
Prevalence of Biomarker	4,4%	Paik et al. 2011
Cost of test	€ 340,67	Standard FISH reimbursement rate in Belgium assumed for all, even if not currently reimbursed
Overall incremental cost of therapy/month (compared to Docetaxel)	€ 8 767	Evaluation report on Xalkori (Dossier 4677.1), INAMI/RIZIV 2012 (available upon request)
Median months of therapy for true + patient	12	Evaluation report on Xalkori (Dossier 4677.1), INAMI/RIZIV 2012 (available upon request)
Median months of therapy for false + patient	6	Assumption based on expert opinion
Incremental benefit of biomarker true + patient (LYG)	0,863	Evaluation report on Xalkori (Dossier 4677.1), INAMI/RIZIV 2012 (available upon request)
Incremental benefit of biomarker false + patient (LYG)	0	
Share of Eligible Population Tested	100%	Assumed

Figure 7 – Impact of changes in specificity on overall ICERs depending on prevalence of the biomarker



In order to better illustrate the importance of the different factors discussed in this chapter, Figure 6 shows a tornado diagram in which baseline values for each variable of the breast cancer example are varied keeping all other inputs constant, in order to assess their impact on the overall ICER results. Scenario 2 was chosen as the departing point (base case) given that it is the only one displaying both suboptimal sensitivity and specificity values (lower than 100%) and thus, the only one which would allow us to test the importance on test accuracy on the overall results. The cost of the therapy (the overall incremental costs of therapy), the cost of the test, the test sensitivity and specificity, and the prevalence of the biomarker were all varied by ± 1%.

As we can see, the test specificity is a very important factor, having a much larger impact on the ICER than the cost of the actual test.

4.4.3 The relevance of test specificity when applied to NGS panels

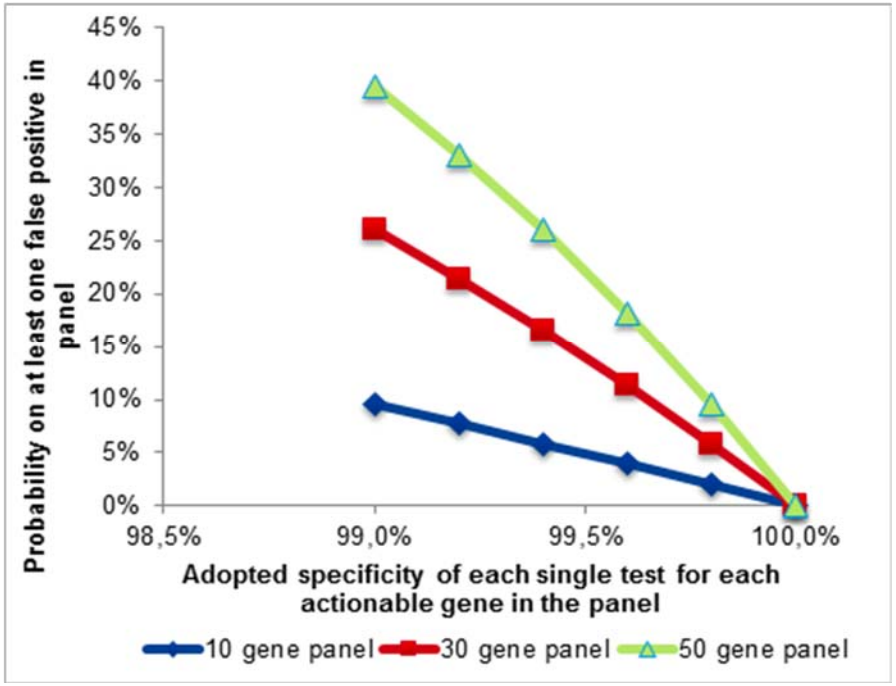
The full value of personalized treatment can only be realized if the patient selection in routine care within a specific health care context is similar to the patient selection in the clinical trials that demonstrated the clinical utility of the combination companion diagnostic plus targeted treatment. The accuracy of the assays used in both settings should therefore be highly similar.

As mentioned above, for FFPE-tissues the loss in specificity of the assay may be related to DNA-fragmentation and non-reproducible sequencing artefacts after PCR amplification.[46] Rare false positives for actionable mutations have been found using NGS panel tests and this may be more likely to occur when the coverage and the number of reads are low and in case of bad quality DNA, as can be seen after fixation and storage. The fact that multiple genes are tested simultaneously in panel tests increases the probability that one or more false positive results are seen. The increase in probability depends on the number of tests performed simultaneously and the probability to obtain a false positive result per gene. A lower coverage of a gene in itself also lowers specificity. These principles explain why there is a higher risk of false positives associated with exome sequencing: the number of genes interrogated is very high and the coverage is low. The need for orthogonal validation in case of exome sequencing is illustrated by recent results by Shi et al.(Shi et al. abstract PD3-4, San Antonio Breast Cancer Conference 2014) They performed whole exome sequencing of 33 specimens from 11 tumor samples and on 8 technical replicates to assess intratumor and intertumor variation relative to technical noise. They found that the within tumor spatial heterogeneity was small and comparable to technical noise. This suggest a high chance of false discovery and necessitates thus orthogonal validation of mutation calls found using exome sequencing. As discussed, the probability of false positives is much lower for NGS panel tests with a high coverage of the specific targets. In clinical practice, one option to reduce the risk of reporting a false positive result is to require confirmation of each actionable mutation with an orthogonal technology before the action/targeted treatment is started.

Many popular variant callers, such as VarScan2 and GATK amongst others, return a probability of significance for each variant position.[48, 74] These probabilities can be used to control the specificity of NGS panel tests. Since NGS panels involve a considerable number of genomic positions, correcting for multiple testing will become essential to control the overall specificity of the actionable mutations that are assessed with the panel. This is illustrated in Figure 7. It shows the impact of the panel size on the probability of making at least one false positive for patients without actionable mutations under the assumption that all actionable genes are independent and when the multiple testing problem is not addressed. Figure 7 shows that the probability on false positives can become very substantial even when high specificities are adopted in each single test for each actionable gene in the panel. The Figure 7 also suggests that the probability of obtaining one or more false positive results can be controlled by adjusting the specificity of each single test. For example, under the assumptions of independence, adopting a specificity >99.90 (99.98) will control the probability on at least one false positive under the 1% level for a panel of 10 (50) actionable genes. This approach corresponds to the well known multiple testing procedure of Hájek and Šidák. The procedure, however, heavily relies on the independence assumption and is known to be rather conservative. In the era of high-throughput analysis multiple testing is a very active area of statistical research and many novel methods have emerged over the last two decades.

Note, however, that correcting for multiple testing to maintain a good overall specificity also affects the sensitivity of the NGS panel test. In clinical practice, it can therefore be beneficial to restrict the downstream analysis of the NGS assay to a limited number of specific genes of the panel according to the particular diagnosis and/or to adopt two-stage procedures. A positive result in the first stage then has to be confirmed with additional sequencing data or with an orthogonal technology before the action/targeted treatment is started.

Figure 8 – Probability of at least one false positive increases with panel size.



Key Points

- **The accuracy of a test (in routine) is important in economic evaluations for test-therapy combinations.**
- **In particular specificity is often noticeably more influential on the overall cost-effectiveness results than the actual price of the test.**
- **Multiple testing has to be addressed when using NGS panel tests to control its overall specificity because many genes are tested simultaneously.**
- **Other factors such as the prevalence of the biomarker also play an important role and should be taken into consideration.**

5 FINANCING OF COMPANION DIAGNOSTICS AND TARGETED THERAPY

5.1 The disconnection between pharmaceuticals and companion diagnostic

5.1.1 The need for a companion diagnostic conflicts with traditional drug development

Pharmaceutical companies traditionally are not used to the co-development and the validation of diagnostic tests. The majority of oncology drugs in the pipeline today however have a companion diagnostic (SCRIP 20140509 p13). It has been stated that the FDA requirements for the validation of companion diagnostics may hamper the development and marketing of new molecules.[75] In March 2014, a series of articles on companion diagnostics were published in a Clinical Cancer Research Focus section, "The Precision Medicine Conundrum: Approaches to Companion Diagnostic Co-development. This series contains contributions from NICE,[76, 77] EMA,[76, 77] and a comparison with the FDA approach.[78] In the EMA contribution the impact of the new EU device regulation for companion diagnostics is also discussed. Discussions on the new EU device regulation are still ongoing. No final consensus view has been formulated so far.

“The possibility of regulating companion diagnostics within the framework of the legislation on medicinal products was initially considered as a possible option. During the public consultation, however, there were concerns with this option, as it might lead to problems for devices that have several intended uses and would need to follow different regulatory regimes. In addition, it was argued that a regulation under the medicinal products legislation would imply higher regulatory burdens and make the diagnostics more dependent on the manufacturer of the medicinal product. Thus, transfer of the responsibility for the assessment of conformity to medicinal product regulatory authorities was rejected. Similarly, regulation of companion diagnostics within a separate centralized or decentralized framework such as exists for pharmaceuticals, was explored. However, a decentralized marketing authorization system based on mutual recognition

was considered to have a significant negative impact on the internal market for medical devices compared with the automatic access associated with CE marking. A central marketing authorization (at the EU level) would require building a new EU public body with sufficiently skilled staff to assess devices, similar to the U.S. Food and Drug Administration. This would have enormous impact on the EU budget, on manufacturers in terms of costs and administrative burden and on innovation in terms of costs for regulatory compliance and time to market." [77]

In the article by NICE,[76] the experience at NICE and other HTA agencies with companion diagnostics is discussed. It is mentioned that the test performance has an impact on the cost-effectiveness but no specific calculations are made. "Although regulation is evolving, to date in Europe, the licensed indication of a pharmaceutical may require the use of a companion diagnostic but the specific test for determining the mutation status is not normally stipulated. This permits the adoption of a diverse range of proprietary and "in-house" tests that are all consistent with the marketing authorization. These different tests can have varying levels of diagnostic accuracy, leading to different patient subpopulations being selected for treatment, with a resulting impact on treatment clinical- and cost-effectiveness. An example is the EGFR tyrosine kinase (EGFR-TK) mutation test."[76]

"A review commissioned by the Australian government identified that an integrated approach was needed to evaluate pharmaceuticals associated with a companion diagnostic. The current methods for health technology assessment in Australia evaluate the drug and associated diagnostic test separately and therefore do not fully capture the benefits of using the therapeutic and test combined. In addition, the review also noted that diagnostic tests generally have a more limited evidence base than therapeutics, which can often make an evaluation of a diagnostic test more methodologically complex and time consuming. This poses a problem for policy makers who need to produce guidance simultaneously on the therapeutic and associated companion diagnostic to ensure patient access. These challenges are not unique to Australia but are faced by many other countries, including the United States, Canada, and many countries in Europe."[76]

"A key issue for many countries is that the experience of evaluating diagnostic technologies is limited compared with that of pharmaceuticals. Payers of pharmaceuticals are often different from payers of diagnostics and the value of a diagnostic may be perceived differently from that of a pharmaceutical, leading to a need for coordinated decision making. The Australian HTAAP and NICE will provide a model to address these issues."[76]

A recent report by the European personalised medicine association (EPEMED) describes the reimbursement approach of targeted treatments and their companion diagnostic in France, Germany, Italy, the United Kingdom and Spain: "Ideally, systematically collected data on actual patient access in terms of drug and companion diagnostic utilization would provide the evidence base for this study. However, such data rarely exist … Pharmaceutical drugs and the associated companion diagnostics are evaluated separately in France, Germany, Italy and Spain. Separate evaluation processes are neither coordinated nor synchronized. In the United Kingdom (England), companion diagnostic evaluation is integrated into the technology appraisal of the associated pharmaceutical drug which avoids delays or inconsistent decisions. Integrated evaluation is therefore recommended for other countries, too." The EPEMED report is available online:
http://www.epemed.org/online/www/content2/104/107/910/pagecontent2/4339/791/ENG/EpemedWhitePaperNOV14.pdf

5.1.2 *Disconnection between reimbursement of companion diagnostics and drugs in Belgium*

Currently, the reimbursement of targeted therapies and their companion diagnostic in Belgium is not connected (neither the procedure nor the budget). There is only an informal sharing of information between the two different administrations that support both procedures.

In Belgium, diagnostic tests are being reimbursed through the nomenclature, a limited list of diagnostic and therapeutic procedures with per provision a description, a key letter according to the medical specialty, a coefficient and a set of application rules. The key letter and the coefficient permit the calculation of the fee. Diagnostics are typically classified as anatomo-pathology (article 32), genetics (article 33) and molecular diagnostics (article 33bis) provisions. The overall budget for art.33 is fixed starting 2013.

Changes or additions of new diagnostic tests to the list have to be proposed by the Technical Medical Council (TMC). The TMC is advised by working groups, in case of diagnostics this is the working group for clinical biology, which is composed of experts in clinical biology, clinicians, and members of the commission of clinical biology of the Belgian Scientific Institute of Public Health. This working group formulates new codes or modifications of existing ones, together with the amount of the fee and an estimation of the resulting budget impact. No legally defined criteria exist for these assessments, which makes this procedure not very transparent. The working group bases its proposals on evidence based medicine, importance in medical practice, and social needs, with a large input coming from the field. The proposal is then being discussed in the plenary meeting of the TMC. When a proposal has been approved by the TMC, it is transferred for approval to the National Committee of Physicians-Insurers (Medico-Mut), the commission for budget control, the insurance committee, the minister of social affairs, finance inspection, the state secretary for budget and the Council of State. At last, decisions are being published as royal decrees, so the King has to sign. There are no time limits and given the series of needed approvals, a procedure takes about 18 months, even more if no budget is available (legal basis: Belgian Law of 1963 on Health and Disability; Belgian Law on Health and Disability, coordinated on July 14th 1994 (articles 22, 27, 28, 35)).

Most of the tests are typically reimbursed through generic codes that describe general laboratory procedures such as 'acquired chromosomal or genetic aberrance in a solid tumour', and are paid based on a technical cost-derived fee schedule. A limited number of tests are specific to a target, indication, and timing in the treatment phase and the fee is higher than for the generic code.

The reimbursement of medicines is regulated through a different procedure (Royal Decree of 21 December 2001). Companies can send applications to the Commission for the Reimbursement of Medicines (CRM), which makes up a proposal regarding the reimbursement of a drug. Based on this proposal, the minister of social affairs takes a motivated decision, which, in case it is positive, needs positive advice from the finance inspection and the state secretary for budget. A positive decision is published in the official journal (het Staatsblad/le Moniteur) as a ministerial order. The criteria for the evaluation of an application are legally established. They include therapeutic value (benefit/risk ratio), social and therapeutic needs, price (with respect to the comparator), pharmaco-economic data, and budgetary impact. The assessment can be regarded as a rapid health technology assessment, since it is strictly bound to time limits: the commission has to send her proposal to the minister within 150 calendar days, after which the minister has 30 more days to make a decision. This timespan can be extended if the company suspends the procedure.

The disconnect between reimbursement procedures and budget entails the following problems (with a growing impact in given that more of those combinations are coming up):

- a reimbursement is provided for the therapy but not for the test
  - because the TMC procedure take much longer then the CRM procedure
  - because the budget for tests is closed and no extra budget is allocated for the new diagnostic test
- an exponential increase of the budget for clinical biology
  - for existing tests
  - as well as for added tests
- it will take much longer to adapt reimbursement of diagnostics to clinical practice changes resulting from evolving knowledge (e.g. recent change of use of *RAS* marker instead of *K-RAS* marker) then it will take to adapt reimbursement of medicines

5.2 Billing codes for molecular diagnostics in oncology in Belgium

5.2.1 *Evolution of billing codes, overall expenditures and testing volumes*

The detection of somatic/acquired genetic/chromosomal changes in tumours was traditionally performed in centers for human genetics and was billed under Article 33 of the nomenclature, which can only be used by these 8 centers. Article 33bis was created in the nomenclature of reimbursed activities to also allow other laboratories to bill molecular tests of human DNA (mainly somatic/acquired, but also e.g. Factor V). The evolution of the test volume and expenditures is given for somatic/acquired molecular tests under Article 33bis. One should take into account that in addition to the volumes in the table, some genetic centers billed some of these tests under Article 33 until it was reworked end of 2012. The number of *in situ* hybridization tests (ISH) for HER2 in breast cancer (588556-588560) nearly doubled between 2008 and 2013 and is now performed in the majority of breast cancer specimen (over 7942 tests for about 10000 new breast cancer cases per year). This is much more than the 24.6% IHC2+/3+ or the 38.3% of samples showing IHC1+/2+/3+ calculated in the KCE report 34. In France a similar absolute number of HER2 ISH tests is performed as in Belgium (http://www.e-cancer.fr/). In France the test is used to confirm 2+ results. The relatively low reimbursement fee for this ISH test in France (130 euros) could help explain the less frequent use compared with the situation in Belgium where this ISH is reimbursed at 340 euros. The expenditure of over 2.6 mio euros for ISH HER2 (plus the costs for IHC) is also important relative to the other tests.

This deserves further investigation as does the large volume of immunoglobulin or T-cell rearrangement tests (1.2 mio euros).

Table 15 – Number of tests per year (year of the activity) and expenditures for selected tests in Article 33bis.

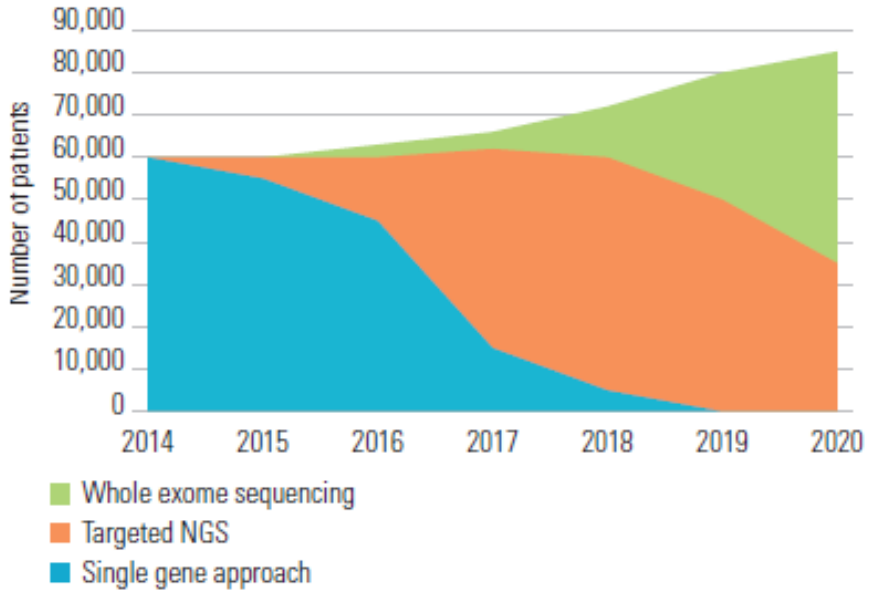
		2008		2009		2010		2011		2012		2013	
Code_pairs		N_Cases	T_Expenses	N_Cases	T_Expenses	N_Cases	T_Expenses	N_Cases	T_Expenses	N_Cases	T_Expenses	N_Cases	T_Expenses
588431_588442	Diagnosis acute leukemia	3.249	€ 367.326	3.084	€ 362.106	4.048	€ 480.280	4.245	€ 507.856	4.162	€ 506.652	4.634	€ 567.702
588453_588464	Diagnosis chronic lymphoid	2.500	€ 276.135	2.634	€ 303.585	3.540	€ 413.003	3.180	€ 374.726	3.523	€ 421.992	4.309	€ 520.572
588475_588486	Ig or T-cel rearranged CLL/NHL	7.568	€ 836.910	7.862	€ 910.007	9.190	€ 1.074.485	9.368	€ 1.104.942	10.472	€ 1.254.454	9.755	€ 1.178.803
588490_588501	Ig or T-cel rearranged other	217	€ 24.305	227	€ 26.767	305	€ 36.474	246	€ 29.335	359	€ 43.527	566	€ 69.206
588512_588523	Diagnosis CML	2.121	€ 233.448	2.290	€ 263.336	2.455	€ 285.440	2.045	€ 240.169	2.010	€ 239.725	1.849	€ 222.921
588534_588545	Diagnosis solid tumor (max 2)	1.619	€ 178.173	3.952	€ 453.809	5.024	€ 585.590	5.894	€ 694.680	8.408	€ 1.006.672	13.560	€ 1.638.886
588556_588560	HER2 ISH breast	3.767	€ 1.141.230	4.570	€ 1.446.925	5.537	€ 1.769.919	6.670	€ 2.152.036	7.725	€ 2.528.386	7.942	€ 2.623.444
588571_588582	non CML monitoring	4.823	€ 532.020	4.819	€ 555.886	5.191	€ 604.975	4.292	€ 505.079	4.509	€ 538.394	5.626	€ 680.068
588593_588604	bcr/abl monitoring	2.802	€ 305.833	3.137	€ 357.453	3.249	€ 374.883	3.445	€ 401.629	3.782	€ 447.318	4.258	€ 508.442
588770_588781	marrow monitoring	54	€ 5.900	58	€ 6.681	36	€ 4.212	6	€ 705	0	€ 0	6	€ 713
589691_589702	JAK2 MPS	0	€ 0	0	€ 0	947	€ 148.630	3.466	€ 548.907	4.072	€ 654.171	4.249	€ 689.379
589713_589724	K-RAS colorectal	0	€ 0	0	€ 0	0	€ 0	1.193	€ 386.888	2.188	€ 717.032	2.540	€ 839.290
Grand Total		30.079	€ 4.104.377	35.113	€ 5.054.160	47.668	€ 6.926.466	53.311	€ 8.268.234	61.379	€ 9.837.960	70.527	€ 11.181.921

The 'generic' codes for acquired genetic/chromosomal changes in solid tumours at diagnosis (588534-588545, also coded as 33b_f in section below) have increased in 2013 to over 1.6 mio euros (may be related to the discontinuation of billing using article 33). If K-RAS tests are added this amounts to nearly 2.5 mio euros.

The budget spent on acquired genetic/chromosomal changes in hemato-oncology diagnosis (not monitoring nor rearrangements, but including JAK2) is about 2 mio euros.

The French cancer centre (INCa) estimates the number of NGS panel tests for somatic mutations needed at 40000 to 60000 per year, as illustrated below.

Figure 9 – Forecast of cancer patients in France tested with molecular tests.



Source: INCa report 2013-14, http://www.e-cancer.fr/

This estimate would translate to a need for the Belgian population of about 7000 to 10 000 given the population size of France is about 6 times that of Belgium. Current article 33bis expenditures for tests that can be replaced by NGS panel tests amount to 2.5 mio euros for solid tumours and 2 mio euros for hemato-oncology. Please note that ALK FISH and some HER2 ISH tests for non-breast cancer are also billed under code 588534-588545 but cannot yet be replaced by NGS DNA panel tests. ALK FISH may however be replaced by RNA tests in the future. The volume of ALK FISH tests in 2013 is assumed to be low.

Some laboratories perform this FISH test in under 10% of NSCLC samples: those that are wild type for EGFR and KRAS and ALK IHC positive. Other laboratories use the FISH test in all EGFR WT samples. Crizotinib was not reimbursed before August 2013. The numbers for activities performed in 2013 were not yet complete as the booking period has not ended yet at the time of writing.

5.2.2 Current issues with billing codes

In this rapidly evolving field of oncology there is a need for the health insurance to cope in an efficient way with new markers, technologies and testing algorithms. Because of the rapid changes in clinically relevant markers and technologies, the current codes and tariffs for reimbursement are quickly outdated. In addition, generic codes do not offer the required transparency to document evolutions in specific marker use over time. Classical examples in the solid oncology tumour area include the EGFR-ALK-ROS1 paradigm in lung cancer. The current nomenclature in article 33bis includes a generic code:

"588534 588545 Opsporen van een verworven chromosoom of genafwijking door middel van een moleculair biologische methode, in de diagnostiche investigatiefase van een niet-lymfoïde en niet-myeloïde vaste tumour B 3000.

588534 588545 Dépistage d'anomalies chromosomiques ou géniques acquises au moyen d'une méthode de biologie moléculaire, dans la phase d'investigation diagnostique d'une tumeur solide non-lymphoïde et non-myéloïde B 3000 »

This generic code can be billed twice for a single diagnostic work-up. This allows for a maximum reimbursement of 124 Euro x 2 = 248 Euro for the

three tests, as there is no specific nomenclature for the EGFR-mutation assay, nor for the ALK FISH or ROS1 FISH test. The amount of 248 euros clearly does not cover the real costs. Furthermore, the number of assays will probably increase in the coming years, rendering higher costs for the laboratory with a continuously fixed reimbursement of 248 Euro to cover all tests, which is nearly impossible.

A similar example concerns RAS-testing in colorectal cancer, where KRAS can be reimbursed using the following nomenclature:

"589713 589724 Opsporen van een verworven afwijking van het K-RASgen door middel van een moleculair biologische methode in het kader van het voorschrijven van een behandeling door tumourspecifieke monoklonale antilichamen bij patiënten met een gemetastaseerd colorectaal carcinoom B 8000

589713 589724 Dépistage d'une anomalie acquise du gène K-RAS par méthode de biologie moléculaire, dans le cadre de la prescription d'un traitement par anticorps monoclonaux spécifiques de la tumeur chez des patients présentant un carcinome colorectal métastasé B 8000. »

However, NRAS is reimbursed using the same generic code mentioned above. If BRAF-testing in colorectal cancer would become part of the required panel (currently there is no evidence) NRAS and BRAF would then need to get reimbursed using the generics code. The amount reimbursed of 248 euros will not cover all costs associated with the testing, unless the patient gets billed.

The examples given illustrate that the current reimbursement system for molecular tests cannot follow this rapidly changing field of health care. A reimbursement should cover the costs of the assay if billing to patients is to be avoided. Transparency and clear communication between the respective government bodies and the laboratories is obligatory in order to maintain a safe cost balance for those laboratories that are able to provide the complete set of tests to the treating physicians.

An important difference between clinical biology tests and article 33bis concerns the procedure for having tests performed by a third party laboratory. For clinical biology (article 24) the transferring lab receives the bill whereas for article 33bis it is permitted that the third party lab bills the tests directly to the health insurance. This principle is the reason why some tests are billed more frequently than allowed: one lab does not know the other has billed the same generic codes already before shipping the sample for additional tests. This principle is also in conflict with the recommendation in KCE report no 20 that laboratories performing molecular tests for oncology should offer the full panel for a given tumour.[6] Service level agreements between hospitals should facilitate that tests are conducted outside of the own hospital.

Current molecular diagnostic tests and the use of article 33bis

The overall list of current molecular diagnostic tests and the use of article 33bis is given in Appendix 4.

The purpose of this table is to clarify the present use of article 33bis. It should demonstrate the complexity of the current situation with respect to the high number of different tests and pathologies, the separate reimbursement codes for diagnostic and follow-up samples, the variety of techniques used, which tests are DNA-tests or RNA-tests, which tests might be replaced by DNA-sequencing with NGS, etc. Finally, the table might help in estimating the required budget for introducing DNA-sequencing .

The table lists most if not all tests that were available at the time article 33bis was introduced (2007) (time 0, time of implementation), as well as the few tests for which a new nomenclature number was added (JAK2 V617F, KRAS; time 1). In addition, as an illustration of the impact of the many newer or future tests, a selection of those was added to the table (time 2). For a more exhaustive list of all new and desired NGS panel tests, see table in Appendix 4.

At time 0, molecular diagnostics was restricted to the demonstration of B/T-cell clonality and single, subentity-specific aberrations (e.g. IGH-BCL2 in FL, BCR-ABL1 in CML, …). As the number of specific aberrations in different lymphoma and acute leukemia subentities were already quite high at that time, two "generic" numbers for gene aberrations in lymphoma and AML/ALL were introduced (588453-588464 and 588431-588442 respectively). As at that time (time 0), also different sarcomas were already known to harbour specific translocations, a generic code for these different tests was also included (588534-588545). As the description of the code is not specific for sarcoma ("solid tumors"), it can also be used for reimbursement of molecular testing in carcinomas, which has only emerged and strongly increased during the last years.

For other pathologies like MDS (without blasts excess) and MPN (not CML) however, molecular profiling has only have become recently of interest, so no (generic) reimbursement codes are included in article 33bis. In addition, the use of the existing generic codes (for lymphoma, AML/ALL and solid tumors) is restricted by a defined maximum frequency (Max 2, 3 or 5 per sample). As a consequence, many tests that are already performed or that will be introduced in the near future, cannot be reimbursed (indicated under "reimbursed in practice" column).

Apart from the limitations of article 33bis, the laboratories are confronted with many technical and practical problems while gradually implementing all new and emerging tests. The conventional "gene-by-gene" analysis, by PCR, Sanger sequencing, Pyrosequencing, … is time-consuming, needs a high amount of DNA, has high turn-around-times (especially if reflex testing is used), and is expensive in the use of reagents, equipment, personnel, validation and quality control. These problems are becoming more and more important, while gradually implementing more and more single gene analyses.

The introduction of a targeted-NGS analysis can partly overcome these problems, as it is a single, multiplex assay, analyzing a broad panel of genes (or gene regions) at once (massively parallel), requiring only a limited amount of DNA. As many different gene tests can be consolidated on the NGS platform, a single workflow can replace many single tests, reducing hands-on-time and turn-around-times. In addition, the panel can be designed as such that genes that might become of clinical relevance in the future are already included in the panel, reducing the future need for additional validation and implementation costs.

However, DNA NGS cannot replace all present tests. See the last two columns in the table. Many tests are RNA-tests, for many tests FISH is expected to remain the preferred technique (at least in the near future), some tests might remain single tests for specific indications (e.g. BRAF V600E in HCL). In general, DNA-NGS will only be the method of choice in case "molecular profiling" of a neoplasia is or will be indicated, like for most solid tumors, AML, ALL, MDS, MPN, CLL, … but probably not for well-defined lymphomas, that are characterized by a single gene aberration (FL, BL). Introducing DNA-NGS for solid tumors may replace current mutation tests on DNA, but is not expected to replace current FISH tests in the near future. For AML, ALL, CLL, MDS, MPN … introducing DNA-NGS is required to make a full profiling at the DNA level technically and practically feasible, but it can at present not replace the many RNA-based (AML, ALL) or FISH tests (CLL, PCM, DLBCL, …).

NGS will also not be indicated for BCR-ABL1 detection to confirm CML diagnosis and certainly not for BCR-ABL1 quantification to monitor CML therapy. In general, for follow-up, NGS will probably not be indicated when a specific, single, molecular marker was demonstrated at diagnosis and can be monitored by a single, sensitive PCR analysis (eg. Translocations in acute leukemia). Other tests might be part of a profiling NGS panel but should also be retained as simple PCR-test. An example of this is JAK2. It should be included in a panel for molecular profiling of different myeloid neoplasms but it should also be available as a single, cheap blood test to discriminate secondary polycythemia from polycythemia vera (subtype of MPN).

In the long-term however, it may be expected that tumors (both solid and hematological) will be profiled both at the DNA and RNA level, either in a single or in two separate massively parallel analyses, as well as for copy number variations, methylation status, etc. In addition, the analysis of several tissue or liquid biopsies, sequentially during the course of the therapy (and not exclusively at diagnosis) might be indicated in the future, at least for some patients. These issues are beyond the scope of the KCE project but this future perspective might be taken into account when deciding on the modalities and the degree of flexibility of the reimbursement strategy to be proposed.

5.3 Molecular tests billed by type of tumour diagnosed in Belgium (2010-2011)

5.3.1 Aims and methods of analysis by tumour type

The aim of this study was to estimate the volume and budget used in Belgium for molecular diagnostics in cancer patients. Analyses focused on a selection of 6 different cancer types diagnosed in 2010 or 2011, for which the studied diagnostics were charged around the incidence date.

The study population was selected from the database of the Belgian Cancer Registry (BCR) and included patients with a specific cancer type diagnosed in 2010 or 2011. Based on the patient specific Identification Number of Social Security (INSS), this database was linked with the administrative databases from the health insurance companies, provided by the Intermutualistic Agency (IMA/AIM).

5.3.2 Selection of tumour types

Tumours were selected based on the International Classification of Diseases-10th edition (ICD-10),[79] except for myelodysplastic syndrome (included in hematological malignancies) for which the selection was based on a combination of morphology and behavior according to the International Classification of Diseases for Oncology-3rd edition (ICD-O-3)[80]

The following inclusion criteria were applied:

- Female breast cancer (ICD-10: C50)
- Colon cancer (ICD-10: C18-C19)
- Rectal cancer (ICD-10: C20)
- Lung cancer (IDC-10: C34)
- Prostate cancer (ICD-10: C61)
- Hematological malignancies :
  - Leukemia (ICD-10: C91-C95)
  - Hodgkin's lymphoma (ICD-10: C81)
  - Non-Hodgkin's lymphoma (ICD-10: C82-C86)
  - Multiple myeloma (ICD-10: C90)
  - Myelodysplastic syndrome (ICD-O-3 morphology: 9980-9992 and behavior: 3)

The following patients were excluded from the analyses :

- Patients without an official Belgian residence;
- Patients with no exact incidence date for the concerned invasive tumour;
- Patients without a known INSS;
- Patients who could not be linked to the health insurance data provided by the IMA/AIM

In order to exclude medical acts that were potentially charged for other malignant lesions, patients with additional invasive tumours diagnosed within 6 months before and 12 months after the incidence date of the studied tumour (except non-melanoma skin cancers) were excluded.

5.3.3 IMA/AIM data

All considered nomenclature codes were selected from the articles 32 (anatomo-pathology), 33 (genetics) and 33bis (molecular diagnostics) and are provided in Appendix 5. For the patients concerned, all records charging one of these nomenclature codes within a time frame of 3 months before and 6 months after the incidence date were retrieved from the IMA/AIM database. This time interval around diagnosis was considered to cover most of the tests performed during the diagnostic investigational phase.

Table 17 shows the numbers of patients and IMA/AIM records that were in- or excluded for the analyses, by each tumour type.

Table 16 – Summary of selected patients and records by tumour type

Tumour type	Initial number of patients (BCR)	Number of tumours after linkage*	Number of tumours after code selection**	Number of IMA/AIM records after linkage
Breast	21,728	20,408	20,054	66,774
Colon	12,018	11,030	6,060	9,431
Rectum	4,798	4,439	2,299	3,434
Lung	15,785	14,795	12,215	24,113
Prostate	17,690	16,650	10,263	12,064
Haematological	10,450	9,915	9,102	38,278

** limited to patients without another invasive tumour within 6 months before and 12 months after the incidence date of the studied tumour, and who could be coupled with IMA/AIM databases*
*** limited to tumours for which at least one selected nomenclature code was charged within 3 months before and 6 months after incidence date*
Source: BCR-IMA data

The budgets allocated for the molecular/genetic tests were derived from the amount of reimbursement as mentioned in the IMA/AIM data.

For the volume analyses, all the selected medical acts were first assigned to one laboratory based on the laboratory identification code (RIZIV/INAMI code) provided within the IMA/AIM data. Priority was given to the place where the medical act was performed (variable ss00085 in IMA/AIM data). After assignment, laboratories (n=143) were regrouped into institutes (n=110) based on the name, address and qualification referenced in the lists of active laboratories during the study period by RIZIV/INAMI (lists of March 2011 and June 2012 ; http://inami.fgov.be/care/nl/labo/history.htm).

As administrative data were used for the analyses, some specificities must be kept in mind when interpreting the results. First, some of those nomenclature codes were introduced, modified or suppressed at a certain point in time. Second, only charged medical acts are provided by the IMA/AIM database. Third, according to the billing rules, medical acts can be charged with a specific nomenclature code several times at the same date and/or at different dates.

5.3.4 Results

Overall, for the seven most frequently charged couples of nomenclature codes (358,903 medical acts), the amount of reimbursement was about 21,048,102.74€. Table 18 summarizes the number of medical acts performed, the percentage of tumours for which at least one of these acts had been charged (at least once) and the total amount of reimbursement per couple of nomenclature codes and tumour type. For these seven couples of nomenclature codes, the coverage rate of tumours with at least one genetic/molecular test charged varied between 51.8% and 98.3% for solid tumours whereas it was 89.8% for hematological malignancies. While code 32_a covers common immunohistochemical tests, 32_b specifically reimburses antibodies for ER, PR, HER2 and EGFR immunohistochemistry, explaining its utilization rate in breast (ER, PR, HER2) and lung (EGFR) cancer. Codes 33b_g (HER2 in situ hybridization) and 33b_i (kRAS testing, since May 2011) are charged within the context of breast and metastasized colorectal cancer, respectively. Besides these tumour-specific codes, 33_m (generic code for DNA hybridisation test performed in a recognised genetic center) and 33b_f (molecular test performed on a solid tumour sample) seemed to be the most frequently charged acts.

Table 17 – Medical acts, percentage of patients and amount of reimbursement by tumour type

	Breast (N=20,408)			Colon (N=11,030)			Rectum (N=4,439)		
Couple of codes	Acts (N)	Tumours (%)	Amount (€)	Acts (N)	Tumours (%)	Amount (€)	Acts (N)	Tumours (%)	Amount (€)
32_a	93 048	94,2	2 179 111	19.764	45,7	477 567	7.250	42,1	172 777
32_b	63 087	88,9	4 482 996	288	1,6	20 977	100	1,6	7 221
33_l	112	0,5	35 471	70	0,6	22 258	21	0,4	6 662
33_m	1 191	3,6	375 542	884	7,2	279 042	303	6,2	95 584
33b_f	198	0,6	23 501	1.474	8,2	173 658	575	7,7	67 528
33b_g	9911	45,6	3 186 339	7	0,1	2 271	-	-	-
33b_i	1	0,0	320	556	4,9	180 800	208	4,6	67 604
All	167 548	98,3	10 283 283	23.043	54,9	1 156 575	8.457	51,8	417 380

	Lung (N=14,795)			Prostate (N=16,650)			Haematological (N=9,915)			All types	
Couple of codes	Acts (N)	Tumours (%)	Amount (€)	Acts (N)	Tumours (%)	Amount (€)	Acts (N)	Tumours (%)	Amount (€)	Acts (N)	Amount (€)
32_a	58 337	79,7	1 394 854	34 870	61,5	794 498	42.425	70,3	991 548	255 694	6 010 358
32_b	4 702	27,4	338 390	20	0,1	1 453	143	1,0	10 249	68 340	4 861 289
33_l	168	1,0	53 572	58	0,3	18 432	10.207	68,4	3 241 137	10 636	3 377 534
33_m	1 679	10,6	532 883	58	0,3	18 271	4.691	35,1	1 489 315	8 806	2.790 639
33b_f	2 334	8,5	275 441	9	0,0	1 032	98	0,8	11 614	4 688	552 777
33b_g	14	0,1	4 510	-	-	-	6	0,1	1 927	9 938	3 195 049
33b_i	34	0,2	11 075	-	-	-	2	0,0	653	801	260 455
All	67 268	82,5	2 610 729	35 015	61,6	833 687	57.572	89,8	5 746 446	358 903	21 048 102

Source: BCR-IMA data

As described further (Table 20), the most frequently charged nomenclature codes for hematological malignancies differed from those for solid tumours.

Focusing on nomenclature codes 33_m and 33b_f at the tumour level, it appeared that for only 0.5% of all tumours with at least one of the selected medical acts performed, both medical acts were charged: this proportion ranged from 0.0% for breast cancer to 1.1% for colon cancer. At the institute level, all the 33_m charging institutes (i.e. the genetic institutes, n=8) charged 56.4% of all 33b_f medical acts for all tumour types together (data not shown), ranging from 38.3% in lung cancer to 95.5% in breast cancer (Table 19). This suggests that institutes tended to preferentially charge one of both nomenclature codes for a particular tumour type, indicating a practice difference among the genetic institutes for charging 33_m and 33b_f nomenclature codes. One should realize that 33_m billings also cover germline mutation testing, which is mainly done for breast cancer patients (BRCA1, BRCA2). For breast cancer, ISH (33b_g) was used for confirmation after HER2 IHC (32_b) in 46% of the cases in the period studied, which could correspond to all IHC 1/2/3+.

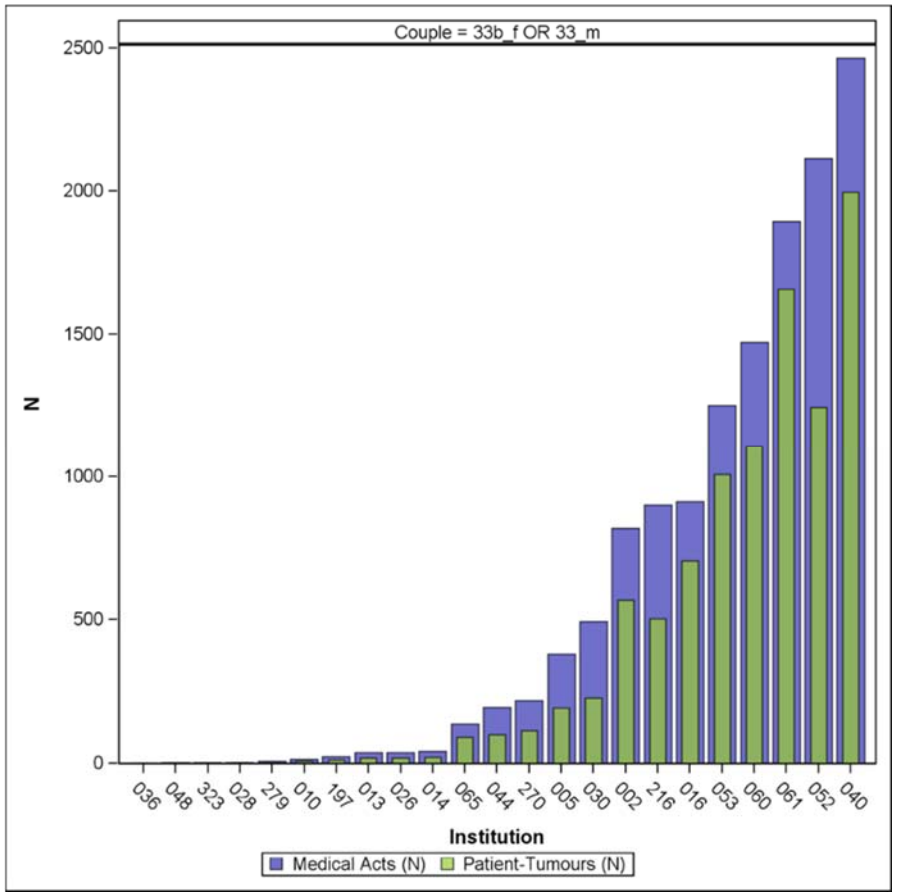
Table 18 – Institutes charging medical act 33b_f in addition to 33_m for breast, colon and lung cancer

Breast cancer	Acts 33_m		Acts 33b_f		Colon cancer	Acts 33_m		Acts 33b_f		Lung cancer	Acts 33_m		Acts 33b_f	
Institutes (33_m)	N	%	N	%	**Institutes (33_m)**	N	%	N	%	**Institutes (33_m)**	N	%	N	%
016	21	1,8	3	1,5	**002**	1	0,1	2	0.1	**052**	5	0,3	595	25,5
060	45	3,8	169	85,4	**030**	9	1,0	-	-	**002**	10	0,6	207	8,9
030	63	5,3	3	1,5	**016**	11	1,2	137	9.3	**030**	40	2,4	4	0,2
002	87	7,3	1	0,5	**052**	44	5,0	435	29.5	**016**	149	8,9	4	0,2
053	115	9,7	2	1,0	**040**	128	14,5	213	14.5	**040**	304	18,1	8	0,3
061	170	14,3	2	1,0	**053**	138	15,6	-	-	**060**	326	19,4	3	0,1
052	239	20,1	9	4,5	**061**	263	29,8	298	20.2	**053**	348	20,7	62	2,7
040	443	37,2	-	-	**060**	288	32,6	-	-	**061**	496	29,5	8	0,3
All	1191	100,0	189	95,5	**All**	884	100,0	1.087	73,7	**All**	1679	100,0	893	38,3

Source: BCR-IMA data

Figure 9 represents the total amount of charged 33_m and/or 33b_f acts and the number of tumours for which these tests were performed by institute, for all studied tumours together. More than the half of all institutes charged less then 500 tests during the observation period. In accordance with the billing guidelines, 33b_f was charged twice for 65% of all tumours tested. In contrast, 81% of all 33_m acts were only charged once for the same tumour. These proportions correspond to the tumour-specific data, except for breast cancer patients for which 59% of all 33b_f acts were charged only once and 61% of all 33_m were charged twice (probably referring to BRCA1 and BRCA2, data not shown).

Figure 10 – Number of patient-tumours and medical acts by institute for 33_m and/or 33b_f



Source : BCR-IMA data

As already mentioned, the nomenclature codes used for molecular diagnostics in hematological malignancies, differed significantly from those used in solid tumours (Table 20).

Table 19 – Medical acts, percentages of tumours and amount of reimbursement for haematological malignancies

Haematological malignancies (N=9,915)			
Couple	Acts (N)	Tumours (%)	Amount (€)
32_a	42 425	70,3	991 548
32_b	143	1,0	10 249
33_l	10 207	68,4	3 241 137
33_m	4691	35,1	1 489 315
33b_a	5062	14,3	606 734
33b_b	3675	17,1	431 502
33b_c	7170	36,5	845 237
33b_d	367	2,0	44 068
33b_e	659	5,7	77 549
33b_f	98	0,8	11 614
33b_h	312	3,0	49 649
33b_j	2184	10,0	258 406
33b_k	448	2,7	52 105

Source: BCR-IMA data

The amount of charged medical acts for each hematological malignancy by selected nomenclature was in line with the billing rules. Seventy-two percent of all 33_l acts and 81% of all 33_m acts were charged only once. In contrast, 33b_a was charged at least 5 times in 667 tumours, representing 47% of the tested cases. Immunohistologic investigations (32_a) were charged four times for 2,962 (42%) and more than five times for 2,930 (42%) tumours with at least one 32_a charged act. Investigations of type 33b_b were charged between one and three times for 1,571 (92%) of the investigated tumours, whereas for 33b_c, 2914 (80%) tumours with this act were either charged once or twice.

Remarks and Comments

As the studied period covers a timeframe of -3/+6 months around the date of diagnosis, the diagnostic investigation phase may not be fully covered. The volume of KRAS testing in colorectal cancer is underestimated. First, this code has only been introduced in May 2011, and secondly, it is dedicated to tumours in metastatic setting (often occurring later in the disease course).

5.4 Current cost of NGS panel tests

For laboratories it may be cheaper to run a small NGS panel instead of testing two or more genes individually with specific molecular tests. In addition, NGS panel tests are still possible on small biopsies with relatively little DNA. The total reagent costs of separate tests based on Sanger sequencing for KRAS, NRAS, BRAF have been shown to be similar to the reagent costs to run an NGS panel.[81] This study evaluated the TAT and costs associated with using an Ion Torrent panel next-generation sequencing AmpliSeq Panel for Lung and Colon Cancer. The TAT was 13.0 working days (range 7-14) and the costs for consumables for testing KRAS, NRAS and BRAF, including seven exons was 196 Euro. For Ion Torrent Sequencing sequencing, as eight barcoded samples were loaded for each 316 chip, the costs for each patient was 187.23 Euro, amounting to a maximum of 262.20 Euro whenever low level library concentration required DNA-amplification. Similarly, initialisation failures, occurring twice for a total of 16 samples, led to an increase of 7.80 Euro per sample. It should be noted that the coverage used in this report was higher than the coverage reported by laboratories in Belgium, explaining the difference in reagent costs with the table below.

Table 21 presents the cost of NGS panel tests based on information provided by companies as well as laboratories. The situation at five Belgian laboratories is illustrated below. The costs are non-audited and were provided directly by the (coded) laboratories to KCE.Since the detail provided varied from one laboratory to another, all data received were grouped under three main headings (i.e. equipment including services, reagents and personnel) in order to facilitate cost comparisons and data

interpretation. Details on the platforms used, the number of genes included in their panel, the number of annual samples in which their calculations were based, and the depreciation period applied to estimate annual equipment costs were available in all cases. All laboratories used an activity based (microcosting) approach, in which prices of consumables and equipment reflected the market's valuation, while personnel costs were derived from gross annual salary data. Estimations were provided from DNA extraction to reporting of results.

The laboratories performing a run once a week, have an intra-laboratory TAT of up to 10 days. The costs will vary mainly based on the technology selected, the coverage, the size of the panel and the TAT (or proportion of incomplete runs), and the depreciation period for the instruments (3 or 5 years).

Table 20 – Cost calculations per sample analysed for actionable mutations using NGS, results for Belgian laboratories.

Laboratory code	Lab A		Lab B	Lab C					Lab D			Lab E							
Instrument platform	Ion Torrent PGM		Illumina	Illumina MiSeq					Illumina MiSeq			Illumina MiSeq							
Panel regulatory status	RUO	RUO	RUO	Custom	Custom	RUO	Custom	RUO	RUO	Custom	RUO	RUO				RUO			
Panel description	Colon-Lung	Cancer	TruSight Tumor	Home made		Trusight Tumor	Agilent Custom	Trusight Myeloid	Trusight Tumor	Cust-Amplicon	TruSight Myeloid	Trusight Tumor				Trusight Myeloid			
Hotspots, kb in panel	15kb		23kb	33 hotspots		23kb	60kb	141kb	23kb	10kb	141kb	23kb				141bp			
Genes in panel (n)	22	50	26	10		26	50	54	26	24	54	26				54			
Samples per year (n)	750	250	1200	624	832	624	624	416	728	729	240	416	624	728	1040	416	624	728	1040
Samples per run (n)	1 to 32	1 to 16	12	12	16	1 to 12	12	8	6 to 8	7 to 12	8	8	8	8	8	8	8	8	8
Runs per year (n)	55 to 65		100	52	52	156	52	52	104	67	30	52	78	91	130	52	78	91	130
Depreciation period (y)	5	5	3	5	5	5	5	5	3	3	3	5	5	5	5	5	5	5	5
Equipment (incl. service)	€ 36	€ 36	€ 71	€ 25	€ 19	€ 75	€ 25	€ 25	€ 56	€ 56	€ 56	€ 92	€ 61	€ 46	€ 37	€ 92	€ 61	€ 46	€ 37
Running reagents	€ 109	€ 169	€ 295	€ 188	€ 166	€ 455	€ 215	€ 311	€ 254	€ 211	€ 215	€ 282	€ 226	€ 199	€ 182	€ 265	€ 210	€ 182	€ 166
Personnel laboratory	€ 60	€ 60	€ 91	€ 47	€ 47	€ 61	€ 101	€ 61	€ 65	€ 55	€ 45	€ 63	€ 45	€ 37	€ 33	€ 63	€ 45	€ 37	€ 33
Total (incl. VAT)	€ 205	€ 265	€ 457	€ 260	€ 232	€ 591	€ 341	€ 397	€ 375	€ 322	€ 316	€ 436	€ 333	€ 282	€ 252	€ 419	€ 316	€ 265	€ 236

The above mentioned costs are an underestimation of the full costs attributable to NGS testing as they do not include the following.

- Cost for interpretation of DNA alterations other than the actionable mutations (considered as clinical research): 60 euros per sample.

- Cost for validation of the assay: estimates vary between laboratories from 15 000 to 42 000 euros. Validation cost per sample: for example 3 to 8 euros per sample if validation used for 5000 samples analysed over 5 years.
- Participation to EQA: 600 euros per EQA scheme plus costs for testing the EQA samples
- Technical audit: 1464 euros for one audit day
- Overhead costs: these costs represent costs not easily attributable to the medical act under evaluation, often because their measurement or registration per unit is challenging. Examples of overhead costs include administration costs, maintenance, depreciation and financial costs for non-medical equipment, heating, catering, management costs, etc.

  The KCE manual for cost-based pricing of hospital interventions[82] recommends to apply a general overhead rate of 56,6% over direct costs, (excluding physicians costs) for medical activities performed in a hospital setting. However, it is unclear whether such rate would be appropriate for laboratory tests. Furthermore, not all laboratories performing molecular tests in oncology are located in a hospital setting. Therefore, these costs have been excluded from our estimations.

  In addition, in hospitals are financed not only using the fees of the nomenclature but also through the Budget Financial Means (BFM). For laboratories in a hospital setting about 80 to 90% of the overall cost will be covered by the nomenclature and 10 to 20% by the BFM. If for example the overall cost for a test (including overhead) would be 500 euros, this means that 400 to 450 euros should be covered by the nomenclature fee.
- The costs associated with the selection of tumor cells for the NGS analysis is included in most cost calculations.

The costs reported above are in line with cost calculations performed in centres in Canada and the UK. In Canada, a laboratory performing a 38 gene panel on 1000 samples per year using an Illumina platform, calculated a cost, excluding validation, of 413 Canadian dollars, about 290 euros. The laboratory in the UK calculated for a 50 gene panel tested in over 1000 samples per year on an Ion Torrent platform an all inclusive cost (including an extensive validation) of 339 BPB, about 410 euros.

Key Points

- **Many EU countries including Belgium lack an integrated reimbursement review of the drug and the companion diagnostic.**
- **Characterisation of tumors using IHC and molecular tests at diagnosis accounts for a yearly budget of over 10 mio euros. Breast cancer accounts for half of the budget, followed by hematological malignancies.**
- **A strong increase in number of tests and budget over the last years is also seen for colorectal cancer and lung cancer, solid tumors where NGS panel test could have a role.**
- **Billing of somatic DNA alterations using article 33 continued in 2010-11 in some genetic centres.**
- **The Article 33bis billing codes cannot cope with the speed of change ongoingh in the field of oncology.**
- **The maximum number of NGS panel tests per year for Belgium is 7000 to 10000, replacing in part many of the current techniques.**
- **The all inclusive cost for a NGS panel test limited to DNA alterations of direct clinical utility varies by platform. It is in the 300 euros to 400 euros range provided 1000 samples per year are tested.**
- **The currently available budget in Article 33bis open for tests that can be performed using a NGS panel is over 4.5 mio euros.**
- **In addition, all expenditures of current testing should be reviewed. For example, another 1.5 mio euros could possibly made available if the HER2 ISH reimbursed rate (340 euro) is aligned with the rate in France (130 euro) and the test is no longer used to confirm HER2 IHC negative samples.**

6 OPTIONS FOR INTRODUCING NGS PANEL TESTS IN THE BELGIAN HEALTHCARE

Advantage, disadvantages, opportunities and risks

The advantages of using gene panels are that these are potentially cost-saving since multiple markers are tested in a single run, rendering a result of multiple variants in a much shorter turnaround time than if each variant is tested separately. In addition, the overall turnaround time can be lower since all the markers are assessed in the same run and there is a more optimal utilisation of the tissues.

Disadvantages of gene panels include the risk of information overload, the lack of clinical validity for every gene tested and importantly the challenge of interpretation of the findings. An additional option facilitated by NGS is the concept of off-label use. This option may be potentially offer added value to the introduction of next generation sequencing in a health care model, if clinical utility is clearly confirmed. Also the budget impact of this evolution may need to be studied.

A recent report on the motivations of hospitals and laboratories to implement next generation sequencing technologies in routine practice has been undertaken in order to understand the process by which such decisions were made. An open survey was conducted among 13 laboratory medical directors and/or department of pathology chairs of academic institutions. There were three main reasons for implementing NGS into their practices, namely

1. More effective delivery of cancer care;
2. The perceived need for institutional leadership in the field of genomics and
3. The perceived premise that NGS will be cost-effective.

The most important barriers that were identified were

1. Challenges in interpreting genetic variants;
2. Establishing the bioinformatics infrastructure and
3. Curating the data from medical, ethical and legal standpoints.

Importantly, the limited availability of information on the outcome was striking. Therefore, it is suggested in this article that setting up metrics for quantitative assessment of patient outcome to be analysed in a prospective manner, test volumes, test menu, financial sustainability, etc… should be installed.[83] This early adopter attitude, as seen for many device innovations, may also be present in many Belgian laboratories, both in university and non-university hospitals.

Education

As many health professionals were trained before molecular diagnostics in oncology were introduced in routine care, there is a more general need for education in this field. Especially pathologists play a crucial role as they are expected to explain the clinical significance of molecular findings during the multidisciplinary team meetings. Genomics-related patient care necessitates a multidisciplinary approach, with the instalment of so called “molecular advisory boards” or “molecular sequencing boards” that includes expert clinicians, molecular pathologists, clinical biologists and geneticists whenever it concerns solid tumour and/or haematological testing; scientists, ethicist and bioinformaticians whenever applicable.

Test accuracy in routine

As documented above, the full value of targeted therapy can only be realized if the patient selection in routine care is more or less identical to the patient selection made in the trials that demonstrated the clinical utility. The accuracy of the companion diagnostics used in both settings should therefore be highly similar. Especially a loss in specificity of the companion diagnostic can result in a drop of cost-effectiveness of the targeted treatment. The same considerations are applicable if the companion diagnostic is based on NGS. It should be taken into account that the more markers are evaluated simultaneously in a panel test (assuming similar coverage) the higher the probability of a false positive result.

Standardisation

International standards should be developed for NGS based tests in clinical routine. There are several international initiatives ongoing that aim to determine NGS-standards for clinical utility that seek to strike a balance between the development of quality evidence for decision-making and rapid assimilation of genomic information for patient care in a health care setting

There is a need for a multidisciplinary National Committee (preferentially embedded in an international consortium) that regularly updates the clinically relevant markers in oncology and interacts with the National Federal Authorities and reimbursement institutions. This could be based on the Belgian model for hereditary genetic testing. Health-economic considerations are important if expensive targeted drugs are used.

Expertise and centralisation

The experience in other countries, namely in the UK and the Netherlands exemplifies that high volume expert and certified genomic centres are needed to comply with all required quality requirements. It is clear that the introduction of NGS in a health care system should be accompanied with the ability of the centre to develop a high quality context at all levels of technicality and during the whole test cycle.

There is a need to develop national uniform guidelines, based on international guidelines so that the use and requests of molecular assays is uniform across centers. This will aid in avoiding discordant practices in different hospitals. Developing such guidelines may be complex and should cover the pre-analytical variables that may impact the result, quality metrics for both the wet and dry components of the NGS test cycle, accompanied by reasonable turnaround times preferentially in a high volume practice with a standardized methodology for variant annotation and reporting.

Health insurance

In this rapidly evolving field of oncology there is a need for the health insurance to cope in an efficient way with new markers, technologies and testing algorithms. These markers are an essential part of the characterisation of tumours reported to the cancer registry. Registration of the test results in a standard and automated way should become routine practice and can be realized if one makes it a condition for test reimbursement as shown in the table below. This would make it possible to know the frequency of specific actionable mutations per tumor type, important to assess the feasibility of clinical trials.

Because of the rapid changes in clinically relevant markers and technologies, the current codes and tariffs for reimbursement are quickly outdated. In addition, generic codes do not offer the required transparency to document evolutions in specific marker use over time.

Therefore, we propose the following system for the financing of selected IHC markers and all molecular markers that are of relevance for the characterisation of tumours during the diagnostic workup. The steps include an obligatory but fully automated registration of the test and the result at the cancer registry. A unique registration number ('ticket') is generated upon registration which becomes part of the data set used for the billing of the test. Also a pathway for obtaining a 'ticket' in case no tumor is confirmed should be foreseen.

Table 21 – Proposed system for the registration and billing of markers for the characterisation of malignancies during the diagnostic workup

steps in registration and reimbursement process	unique ticket confirming registration (sequential number generated upon registration)	ID of oncology center	ID of lab performing test	technology class (IHC, ISH, PCR, NGS small panel, NGS large panel,...) nomenclature code determining reimbursed amount based on activity-based cost	detailed test ID (HER2 ISH, ALK FISH, NRAS, BRAF V600,..) can be pseudocode	test result in standardised format
step 1		request for test by oncology center to lab				
step 2		result obtained at oncology center from lab				
step 3		result reported by oncology center to cancer registry				
step 4	unique ticket confirming registration automatically sent back to oncology center					
step 5	oncology center bills the test including the unique ticket					
step 6	reimbursement by health insurance agency, after check					

In this concept the pathologist/clinical biologist/geneticist at the hospital the patient is seen and diagnosed and the sample is stored (the oncology center in the table above) is in control of ordering the tests at the hospital laboratory in the local hospital or to ship the sample for testing at an external laboratory. This external laboratory will send the bill to the ordering hospital, as is current standard practice for clinical biology tests. This way one avoids that tests are performed (and billed) multiple times at different laboratories.

An important difference between clinical biology tests and article 33bis concerns the procedure for having tests performed by a third party laboratory. For clinical biology (article 24) the transferring lab receives the bill whereas for article 33bis it is permitted that the third party lab bills the tests directly to the health insurance. This principle is the reason why some tests may be billed more frequently than allowed: one lab does not know the other has billed the same generic codes already before shipping the sample

for additional tests. This principle is also in conflict with the recommendation in KCE report no 20 that laboratories performing molecular tests for oncology should offer the full panel for a given tumour.[6] Service level agreements between hospitals should facilitate that tests are conducted outside of the own hospital.

The financing system should reward the appropriate collection, storage, and (if needed) shipment of the sample as well as the use of an appropriate testing algorithm. Therefore it is suggested a lump sum is provided for the pathologist/clinical biologist/geneticist preparing/shipping the sample and another fee for the selection of tumor cells for analysis and interpretation of the tests (IHC, ISH, cytogenetic, PCR, NGS, …) during the diagnostic evaluation of a new cancer. In addition to the lump sum, an amount should be paid for each test performed. In order to stimulate the use of the most cost-effective diagnostic algorithm and to avoid overuse of tests, the amount paid per test should cover the actual cost, and this amount should be re-evaluated on a regular base as technology platforms change.

APPENDICES

APPENDIX 1. GLOSSARY

Sources (with permission):

- http://www.genomicsengland.co.uk/the-100000-genomes-project/glossary/
- *'Next-Generation DNA Sequencing Informatics' (2013) Edited by Stuart M. Brown,* New York University School of Medicine (*ISBN 978-1-936113-87-3)*

Algorithm	A step-by-step method for solving a problem (a recipe). In bioinformatics, it is a set of well-defined instructions for making calculations. The algorithm can then be expressed as a set of computer instructions in any software language and implemented as a program on any computer platform.
Alignment	Arranging different sequences of DNA (usually with a reference sequence) to compare and identify sections where the order of bases might be the same.
Allele	In genetics, an allele is an alternative form of a gene, such as blue versus brown eye color. However, in genome sequencing, an allele is one form of a sequence variant that occurs in any position on any chromosome, or a sequence variant on any sequence read aligned to the genome—regardless of its effect on phenotype, or even if it is in a gene. In some cases, "allele" is used interchangeably with the term genotype.
Amino acids	A set of 20 different molecules that are the basic units of proteins.
Amplicon	An amplicon is a specific fragment or locus of DNA from a target organism (or organisms), generally 200–1000 bp in length, copied millions of times by the polymerase chain reaction (PCR). Amplicons for a single target (i.e., a reaction with a single pair of PCR primers) can be prepared from a mixed population of DNA templates such as human immunodeficiency virus particles extracted from a patient's blood or total bacterial DNA isolated from a medical or an environmental sample. The resulting deep sequencing provides detailed information about the variants at the target locus across the population of different DNA templates. Amplicons produced from many different PCR primers on many different DNA samples can be combined (with the aid of multiplex barcodes) into a single DNA sequencing reaction on an NGS machine.
Annotation	Describing and adding meaning to variants found in a sequenced genome.
Base	The basic building blocks of DNA. There are four bases: adenine (A), cytosine (C), guanine (G) and thymine (T).
Base calling	Predicting the sequence of bases in data produced by a specific type of automated DNA sequencer.
Base pair (bp)	Two bases held together by weak chemical bonds. They run along the length of DNA specifically so that A is always paired with T and C is always paired with G. If you imagine the DNA structure as a twisted ladder, then base pairs are the rungs on the ladder.

BED file	BED is an extremely simple text file format that lists positions on a reference genome with respect to chromosome ID and start and stop positions. NGS reads can be represented in BED format, but only with respect to their position on the reference genome; no information about sequence variants or base quality is stored in the BED file.
Binary Alignment Map (BAM)	A coded, easy to store and accessible version of an SAM.
Bioinformatics	A discipline that uses advanced computer technology to organise, analyse, and interpret biological data. It combines principals from multiple fields such as computer science, mathematics and statistics. Someone who works in this field is called a bioinformatician.
BLAST	The Basic Local Alignment Search Tool was developed by Altschul and other bioinformaticans at the NCBI to provide an efficient method for scientists to use similarity-based searching to locate sequences in the GenBank database. BLAST uses a heuristic algorithm based on a hash table of the database to accelerate similarity searches, but it is not guaranteed to find the optimal alignment between any two sequences. BLAST is generally considered to be the most widely used bioinformatics software.
Capillary DNA sequencing	This is a method used in DNA sequencing machines manufactured by Life Technologies Applied Biosystems. The technology is a modification of Sanger sequencing that contains several innovations: the use of fluorescent labeled dye terminators (or dye primers), cycle sequencing chemistry, and electrophoresis of each sample in a single capillary tube containing a polyacrylamide gel. High voltage is applied to the capillaries causing the DNA fragments produced by the cycle sequencing reaction to move through the polymer and separate by size. Fragment sizes are determined by a fluorescent detector, and the bases that comprise the sequence of each sample are called automatically.
Cell	The basic building block of all living things. All cells are surrounded by a membrane.
ChIP-seq	Chromatin immunoprecipitation sequencing uses NGS to identify fragments of DNA bound by specific proteins such as transcription factors and modified histone subunits. Tissue samples or cultured cells are treated with formaldehyde, which creates covalent cross-links between DNA and associated proteins. The DNA is purified and fragmented into short segments of 200–300 bp, then immunoprecipitated with a specific antibody. The cross-links are removed, and the DNA segments are sequenced on an NGS machine (usually Illumina). The sequence reads are aligned to a reference genome, and protein-binding sites are identified as sites on the genome with clusters of aligned reads.
Chromosome	One DNA molecule that is organised into a thread-like package. Humans have 46 chromosomes in total, we get 23 from our mother and 23 from our father. They are all located in the nucleus. If you think of genetics as the book of life, then DNA is the letters, genes are the words and chromosomes are the chapters.
Clinical Interpretation	Studying sequenced genomes and trying to find out how the results may affect human health and disease.
Cloning	In the context of DNA sequencing, DNA cloning refers to the isolation of a single purified fragment of DNA from the genome of a target organism and the production of millions of copies of this DNA fragment. The fragment is usually inserted into a cloning vector, such as

	a plasmid, to form a recombinant DNA molecule, which can then be amplified in bacterial cells. Cloning requires significant time and hands-on laboratory work and creates a bottleneck for traditional Sanger sequencing projects.
Coding DNA	Sections of DNA that code for protein; coding DNA is your genes. In simple terms, it's the bits of DNA that do stuff we know about. It's just 1% of your total DNA.
Codon	A group of three bases that code for one of the 20 different amino acids. For example 'AGC' codes for an amino acid called Serine.
Consensus sequence	When two or more DNA sequences are aligned, the overlapping portions can be combined to create a single consensus sequence. In positions where all overlapping sequences have the same base (a single column of the multiple alignment), that base becomes the consensus. Various rules may be used to generate the consensus for positions where there are disagreements among overlapping sequences. A simple majority rule uses the most common letter in the column as the consensus. Any position where there is disagreement among aligned bases can be written as the letter N to designate "unknown." There is also a set of IUPAC ambiguity codes (YRWSKMDVHB) that can be used to specify specific sets of different DNA bases that may occupy a single position in the consensus.
Contig	A contiguous stretch of DNA sequence that is the result of assembly of multiple overlapping sequence reads into a single consensus sequence. A contig requires a complete tiling set of overlapping sequence reads spanning a genomic region without gaps.
Companion diagnostic	Diagnostic test specifically carried out for a particular treatment decision, in particular to identify sub-populations of patients for whom treatment is likely to be more effective or safer.
Copy Number Variation (CNV)	A type of variation found in genomes. It may be when there are additional copies of a particular DNA sequence (duplications) or when genetic material is lost (deletions).
Coverage	The number of sequence reads in a sequencing project that align to positions that overlap a specific base on a target genome, or the average number of aligned reads that overlap all positions on the target genome.
De novo sequencing	The sequencing of the genome of a new, previously unsequenced organism or DNA segment. This term is also used whenever a genome (or sequence data set) is assembled by methods of sequence overlap without the use of a known reference sequence. De novo sequencing might be used for a region of a known genome that has significant mutations and/or structural variation from the reference.
De novo variants	De novo simply means 'new'. A de novo variant is one that isn't inherited but appears randomly. So with this type of variant, there is no family history of disease. Its effects may be apparent from birth or appear later and it is disease causing.
Depth/Depth of coverage	The number of times a given base is read by a sequencing machine. 30x sequencing depth means that each base is read on average 30 times. Some bases will be read frequently while others may effectively have no coverage. To get high quality data, you need the greatest depth possible.
Diagnostic Assay	A scientific test used to investigate the cause of a disease.

Diploid	When a cell or organism is diploid, it contains two sets of chromosomes (46 in total), one set from each parent. Somatic cells are diploid.
DNA	Deoxyribonucleic acid, the chemical molecule in our cells that carries genetic information. It is made up of two strands of nucleotides arranged in a spiral to give the double helix structure of DNA.
DNA fragment	A small piece of DNA, often produced by a physical or chemical shearing of larger DNA molecules. NGS machines determine the sequence of many DNA fragments simultaneously.
DNA Methylation	When molecules called methyl groups, made up of one carbon and three hydrogens, are added to a certain bases along a DNA sequence. This modification is important in many ways for genes to function correctly. (see Epigenetics)
Epigenetic studies	The study of changes that can be transferred upon cell division but without a change in the primary DNA sequence.
Epigenome	As well as the complete DNA sequence, this includes all chemical modifications that are added on to DNA affecting its activity in the genome.
Exome	Only 1-2% of the genome contains genes that code for proteins. That 1-2% is called the exome and is collectively made up of exons. The remaining 99% of a genome is called the non-coding DNA regions
Exon	The part of a gene that is converted into proteins. .
FASTA format	This is a simple text format for DNA and protein sequence files developed by William Pearson in conjunction with his FASTA alignment software. The file has a single header line that begins with a “>” symbol followed by a sequence identifier. Any other text on the first line is also considered the header, and any text following the first carriage return/line feed is considered part of the sequence. Multiple sequences can be stored in the same text file by adding additional header lines and sequences after the end of the first sequence.
FASTQ file	A text file format for NGS reads that contains both the DNA sequence and quality information about each base. Each sequence read is represented as a header line with a unique identifier for each sequence read and a line of DNA bases represented as text (GATC), which is very similar to the FASTA format. A second pair of lines is also present for each read, another header line and then a line with a string of ASCII symbols, equal in length to the number of bases in the read, which encode the PHRED quality score for each base.
Fragment assembly	To determine the complete sequence of a genome or large DNA fragment, short sequence reads must be merged. In Sanger sequencing projects, overlaps between sequence reads are found and aligned by similarity methods, then consensus sequences are generated and used to create contigs. Eventually a complete tiling of contigs is assembled across the target DNA. In NGS, there are too many sequence reads to search for overlaps among them all (a problem with exponential complexity). Alternate algorithms have been developed for de novo assembly of NGS reads, such as de Bruijn digraphs, which map all reads to a common matrix of short k-mer sequences (a problem with linear complexity).
GenBank	The international archive of DNA and protein sequence data maintained by the National Center for Biotechnology Information (NCBI), a division of the U.S. National Library of Medicine. GenBank is part of a larger set of online scientific databases maintained by the

	NCBI, which includes the PubMed online database of published scientific literature, gene expression, sequence variants, taxonomy, chemicals, human genetics, and many software tools to work with these data.
Gene	A particular sequence of bases located on a section of a chromosome. Most genes code for proteins and are passed down from parents to their children.
Gene expression	When genes are switched on and influence partucular processes in cells..
Genetic Marker	A piece of DNA sequence that can easily be identified. They're special because we know exactly where they can be found on a chromosome. In genetic research, scientists use them to tell apart different cells, individuals, or species.
Genetics	A discipline that studies the inheritance of similarities and differences between offspring.
Genome	The total DNA sequence of an organism. This includes both the non-coding DNA regions and the exome. From bacteria to broccoli, all living things have unique genomes.
Genome-wide association studies (GWAS)	A study used to associate genetic variations with diseases. It is done by looking for genetic markers in the genomes of many different people.
Genomic medicine	Using genomics to find out more about why people get certain diseases, what drugs might work best for them and coming up with ideas for the development of new drugs.
Genomics	Studying the whole genome and how all the genes might work. It has also come to have a broader meaning to include the laboratory and computing technologies that have been developed because of it.
Genotype	The collection of genes in an individual i.e. their genetic make-up.
Germ line	Egg and sperm cells that pass on genes from parent to child during reproduction.
Haploid	One set of chromosomes, 23 in total. Gametes are haploid.
Haplotype	A set of genetic variants in an individual that are usually found together.
Heterozygote	Humans and most other eukaryotes are diploid, meaning that they carry two copies of each chromosome in every somatic cell. Therefore, each individual carries two copies of each gene, one inherited from each parent. If the two copies of the gene are different (i.e., different alleles of that gene), then the person is said to be a heterozygote for that gene. A homozygote has two identical copies of that gene. In genome sequencing, every base of every chromosome can be considered as a separate data point; thus any single base can be genotyped as heterozygous or homozygous in that individual.
Heterozygous	When a person has inherited two different alleles for a particular gene. For instance a child might inherit one brown eye allele from their mother, and one blue from their father.

High-performance computing (HPC)	High-performance computing (HPC) provides computational resources to enable work on challenging problems that are beyond the capacity and capability of desktop computing resources. Such large resources include powerful supercomputers with massive numbers of processing cores that can be used to run high-end parallel applications. HPC designs are heterogeneous, but generally include multicore processors, multiple CPUs within a single computing device or node, graphics processing units (GPUs), and multiple nodes grouped in a cluster interconnected by high-speed networking systems. The most powerful current supercomputers can perform several quadrillion (1015) operations per second (petaflops). Trends for supercomputing architecture are for greater miniaturization of parallel processing units, which saves energy (and reduces heat), speeds message passing, and allows for access to data in shared memory caches.
High-throughput	When technologies are high-throughput, they are usually automated machinery that can process large amounts of samples at a fast rate.
Homologous	If two DNA sequences from different individuals are homologous, they evolved from a common ancestor.
Homozygous	When a person has inherited two of the same alleles for a given gene. For instance a child might inherit two blue eye alleles, one from their mother and one from their father.
Human Genome Project (HGP	An international effort including 20 sequencing centers in China, France, Germany, Great Britain, Japan, and the United States, coordinated by the U.S. Department of Energy and the National Institutes of Health, to sequence the entire human genome. The effort formally began in 1990 with the allocation of funds by Congress and the development of high-resolution genetic maps of all human chromosomes. The project was formally completed in two stages, the “working draft” genome in 2000 and the “finished” genome in 2003. The 2003 version of the genome was declared to have fewer than one error per 10,000 bases (99.99% accuracy), an average contig size of >27 million bases, and to cover 99% of the gene-containing regions of all chromosomes. In addition, the HGP was responsible for large improvements in DNA sequencing technology, mapping more than 3 million human SNPs, and genome sequences for Escherichia coli, fruit fly, and other model organisms.
Illumina sequencing	The NGS sequencing method developed by the Solexa company, then acquired by Illumina Inc. This method uses “sequencing by synthesis” chemistry to simultaneously sequence millions of ~300-bp-long DNA template molecules. Many sample preparation protocols are supported by Illumina including whole-genome sequencing (by random shearing of genomic DNA), RNA sequencing, and sequencing of fragments captured by hybridization to specific oligonucleotide baits. Illumina has aggressively improved its system through many updates, at each stage generally providing the highest total yield and greatest yield of sequence per dollar of commercially available DNA sequencers each year, leading to a dominant share of the NGS market. Machines sold by Illumina include the Genome Analyzer (GA, GAII, GAIIx), HiSeq, and MiSeq. At various times, with various protocols, Illumina machines have produced NGS reads of 25, 36, 50, 75, 100, and 150 bp as well as paired-end reads.
Indels	Insertions or deletions in one DNA sequence with respect to another. Indels may be a product of errors in DNA sequencing, the result of alignment errors, or true mutations in one sequence with respect to another—such as mutations in the DNA of one patient with respect to the reference genome. In the context of NGS, indels are detected in sequence reads after alignment to a reference genome.

	Indels are called in a sample (i.e., a patient's genome) after variant detection has established a high probability that the indel is present in multiple reads with adequate coverage and quality, and not the result of errors in sequencing or alignment.
Inherit	Receiving genetic information from a parent or ancestor.
Intron	A section of a gene that does not code for proteins i.e. the non-coding DNA. They are called 'introns' because they are 'in between' the coding DNA regions.
ktup, k-tuple, or k-mer	A short word composed of DNA symbols (GATC) that is used as an element of an algorithm. A sequence read can be broken down into shorter segments of text (either overlapping or non-overlapping words). The length of the word is called the ktup size. Very fast exact matching methods can be used to find words that are shared by multiple sequence reads or between sequence reads and a reference genome. Word matching methods can use hash tables and other data structures that can be manipulated much more efficiently by computer software than sequence reads represented by long text strings.
Massively parallel sequencing	A technique where thousands or millions of sequencing processes are run all at once. This allows scientists to analyse large amounts of samples very quickly.
Mate-pair sequencing	Mate-pair sequencing is similar to paired-end sequencing; however, the size of the DNA fragments used as sequencing templates are much longer (1000–10,000 bp). To accommodate these long template fragments on NGS platforms such as Illumina, additional sample preparation steps are required. Linkers are added to the ends of the long fragments, then the fragments are circularized. The circular molecules are then sheared to generate new DNA fragments at an appropriate size for construction of sequencing libraries (200–300 bp). From this set of sheared fragments, only those fragments containing the added linkers are selected. These selected fragments contain both ends of the original long fragment. New primers are added to both ends, and standard paired-end sequencing is performed. The orientation of the paired sequence reads after mapping to the genome is opposite from a standard paired-end method (outward facing rather than inward facing). Mate-pair methods are particularly valuable for joining contigs in de novo sequencing and for detecting translocations and large deletions (structural variants).
Messenger RNA (mRNA)	A molecule that contains the codes for making proteins during the process of translation.
Microarray	A collection of specific oligonucleotide probes organized in a grid pattern of microscopic spots attached to a solid surface, such as a glass slide. The probes contain sequences from known genes. Microarrays are generally used to study gene expression by hybridizing labeled RNA extracted from an experimental sample to the array, and then measuring the intensity of signal in each spot. Microarrays can also be used for genotyping by creating an array of probes that match alternate alleles of specific sequence variants.
Multiple alignment	A computational method that lines up, as a set of rows of text, three or more sequences (of DNA, RNA, or proteins) to maximize the identity of overlapping positions while minimizing mismatches and gaps. The resulting set of aligned sequences is also known as a multiple alignment. Multiple alignments may be used to study evolutionary information about the conservation of bases at specific positions in the same gene across different organisms or about the conservation of regulatory motifs across a set of genes. In NGS, multiple alignment methods are used to reduce a set of overlapping reads that have been mapped to a region of a reference genome

	by pairwise alignment, to a single consensus sequence; and also to aid in the de novo assembly of novel genomes from sets of overlapping reads created by fragment assembly methods.
Next-generation (DNA) sequencing (NGS)	DNA sequencing technologies that simultaneously determine the sequence of DNA bases from many thousands (or millions) of DNA templates in a single biochemical reaction volume. Each template molecule is affixed to a solid surface in a spatially separate location, and then amplified to increase signal strength. The sequences of all templates are determined in parallel by the addition of complementary nucleotide bases to a sequencing primer coupled with signal detection from this event.
Noise	Background results produced by a machine that may make it difficult to interpret sequenced genome data.
Non-coding DNA	Sections of DNA that do not code for proteins but still may have a role to play in how your genes are expressed. In simple terms, this 'dark DNA' is the bits we once thought were junk but now know to be important. For example, some of these sections may control which genes are switched on or off.
Nucleotide	Molecules made up of a sugar, a phosphate group and a one of the four bases.
Paired-end read	See Paired-end sequencing.
Paired-end sequencing	A technology that obtains sequence reads from both ends of a DNA fragment template. The use of paired-end sequencing can greatly improve de novo sequencing applications by allowing contigs to be joined when they contain read pairs from a single template fragment, even if no reads overlap. Paired-end sequencing can also improve the mapping of reads to a reference genome in regions of repetitive DNA (and detection of sequence variants in those locations). If one read contains repetitive sequence, but the other maps to a unique genome position, then both reads can be mapped.
Panels	Scientists use panels to study a selection of targeted genes. This helps to narrow down the search window for mutations in genes that might cause disease.
Personalised Medicine	Using specific information about a person's genes and other relevant factors to help decide the most effective treatments. For example in cancer, personalized medicine can use information about a person's tumour to help diagnose, plan treatment and find out how well treatment is working.
Phenotype	A person's physical characteristics that have been determined by their genes, for example brown eyes. This can differ from their genotype. For example, person with brown eyes could still have one blue eye gene and one brown eye gene but because brown eyes are dominant, that is what shows up in their appearance.
Phred score	The Phred software was developed by Phil Green and coworkers working on the Human Genome Project to improve the accuracy of base calling on ABI sequencers (using fluorescent Sanger chemistry). Phred assigns a quality score to each base, which is equivalent to the probability of error for that base. The Phred score is the negative log (base 10) of the error probability; thus a base with an accuracy of 99% receives a Phred score of 20. Phred scores have been adopted as the measure of sequence quality by all NGS manufacturers, although the estimation of error probability is done in many different ways (in some cases with questionable validity).

Polymerase Chain Reaction (PCR)	A method used to amplify samples of DNA so that there is more genetic material for scientists to study.
Proteins	Large, complex molecules that carry out a variety of essential functions in the body. They are made up of polypeptides.
Pyrosequencing	A method of DNA sequencing developed in 1996 by Nyrén and colleagues that directly detects the addition of each nucleotide base as a template is copied. The method detects light emitted by a chemiluminescent reaction driven by the pyrophosphate that is released as the nucleotide triphosphate is covalently linked to the growing copy strand. Each type of base is added in a separate reaction mix, but terminators are not used; thus a series of identical bases (a homopolymer) creates multiple covalent linkages and a brighter light emission. This chemistry is used in the Roche 454 sequencing machines.
Reference genome	A curated consensus sequence for all of the DNA in the genome (all of the chromosomes) of a species of organism. Because the reference genome is created as the synthesis of a variety of different data sources, it may occasionally be updated; thus a particular instance of that reference is referred to by a version number.
Reference sequence	The formally recognized, official sequence of a known genome, gene, or artificial DNA construct. A reference sequence is usually stored in a public database and may be referred to by an accession number or other shortcut designation, such as human genome hg19. An experimentally determined sequence produced by a NGS machine may be aligned and compared to a reference sequence (if one exists) in order to assess accuracy and to find mutations.
Repetitive DNA	DNA sequences that are found in identical duplicates many times in the genome of an organism. Some repetitive DNA elements are found in genomic features such as centromeres and telomeres with important biological properties. Other repetitive elements such as transposons are similar to viruses that copy themselves into many locations on the genome. Simple sequence repeats are another type of repetitive element comprised of linear repeats of 1-, 2-, or 3-base patterns such as CAGcagCAGcag.… A short sequence read that contains only repetitive sequence may align to many different genomic locations, which creates problems with de novo assembly, mapping of sequence fragments to a reference genome, and many related applications.
RNA-seq	The sequencing of cellular RNA, usually as a method to measure gene expression, but also used to detect sequence variants in transcribed genes, alternative splicing, gene fusions, and allele-specific expression. For novel genomes, RNA-seq can be used as experimental evidence to identify expressed regions (coding sequences) and map exons onto contigs and scaffolds.
Roche 454 Genome Sequencer	DNA sequencers developed in 2004 by 454 Life Sciences (subsequently purchased by Roche) were the first commercially available machines that used massively parallel sequencing of many templates at once. These “next-generation sequencing” (NGS) machines increased the output (and reduced the cost) of DNA sequencing by at least three orders of magnitude over sequencing methods that used Sanger chemistry, but produced shorter sequence reads. 454 machines use beads to isolate individual template molecules and an emulsion PCR system to amplify these templates in situ, then perform the sequencing reactions in a flow cell that contains millions of tiny wells that each fits exactly one bead. 454 uses pyrosequencing chemistry, which has very few base-substitution errors, but a tendency to produce insertion/deletion errors in stretches of homopolymer DNA.
SAM/BAM	See BAM file.

Sanger sequencing method	The method developed by Frederick Sanger in 1975 to determine the nucleotide sequence of cloned, purified DNA fragments. The method requires that DNA be denatured into single strands, then a short oligonucleotide sequencing primer is annealed to one strand, and DNA polymerase enzyme extends the primer, adding new complementary deoxynucleotides one at a time, creating a copy of the strand. A small amount of a dideoxynucleotide is included in the reaction, which causes the polymerase to terminate, creating truncated copies. In a reaction with a single type of dideoxynucleotide, all fragments of a specific size will end with the same base. Four separate reactions containing a single dideoxynucleotide (ddG, ddA, ddT, and ddC) must be conducted, and then all four reactions are run on four adjacent lanes of a polyacrylamide gel. The actual sequence is determined from the length of the fragments, which correspond to the position where a dideoxynucleotide was incorporated.[2]
Sequence alignment	An algorithmic approach to find the best matching of consecutive letters in one sequence (text symbols that represent the polymer subunits of DNA or protein sequences) with another. Generally sequence alignment methods balance gaps with mismatches, and the relative scoring of these two features can be adjusted by the user.
Sequence Alignment Map (SAM)	The file that is created after sequencing and alignment of genomes. The file also has headers to explain the information provided for example, 'position of variant'.
Sequence assembly	A computational process of finding overlaps of identical (or nearly identical) strings of letters among a set of sequence fragments and iteratively joining them together to form longer sequences.
Sequence fragment	A short string of text that represents a portion of a DNA (or RNA) sequence. NGS machines produce short reads that are sequence fragments that are read from DNA fragments.
Sequence read, short read	When DNA sequence is obtained by any experimental method, including both Sanger and next-generation methods, the data are obtained from individual template molecules as a string of nucleotide bases (represented by the letter symbols G, A, T, C). This string of letters is called a sequence read. The length of a sequence read is determined by the technology. Sanger reads are typically 500–800 bases long, Roche 454 reads 200–400 bases, and Illumina reads may be 25–200 bases (depending on the model of machine, reagent kit, and other variables). Sequence reads produced by NGS machines are often called short reads.
Sequence variants	Differences at specific positions between two aligned sequences. Variants include single-nucleotide polymorphisms (SNPs), insertions and deletions, copy number variants, and structural rearrangements. In NGS, variants are found after alignment of sequence reads to a reference genome. A variant may be observed as a single mismatched base in a single sequence read, or it may be confirmed by variant detection software from multiple sources of data.
Sequencing	Reading along DNA to determine the order of every base pair, letter by letter.
Sequencing by synthesis	This is the term used by Illumina to describe the chemistry used in its NGS machines (Illumina Genome Analyzer, HiSeq, MiSeq). The biochemistry involves a single-stranded template molecule, a sequencing primer, and DNA polymerase, which adds nucleotides one by one to a DNA strand complementary to the template. Nucleotides are added to the templates in separate reaction mixes for each type of base (GATC), and each synthesis reaction is accompanied by the emission of light, which is detected by a camera. Each

	nucleotide is modified with a reversible terminator, so that only one nucleotide can be added to each template. After a cycle of four reactions adding just one G, A, T, or C base to each template, the terminators are removed so that another base can be added to all templates. This cycle of synthesis with each of the four bases and removal of terminators is repeated to achieve the desired read length.
Sequencing primer	A short single-stranded oligonucleotide that is complementary to the beginning of a fragment of DNA that will be sequenced (the template). During sequencing, the primer anneals to the template DNA, then DNA polymerase enzyme adds additional nucleotides that extend the primer, forming a new strand of DNA complementary to the template molecule. DNA polymerase cannot synthesize new DNA without a primer. In traditional Sanger sequencing, the sequencing primer is complementary to the plasmid vector used for cloning; in NGS, the primer is complementary to a linker that is ligated to the ends of template DNA fragments.
SFF file	Standard Flowgram Format is a file type developed by Roche 454 for the sequencing data produced by their NGS machine. The SFF file contains both sequence and quality information about each base. The format was initially proprietary, but has been standardized and made public in collaboration with the international sequence databases. SFF is a binary format and requires custom software to read it or convert it to human-readable text formats.
Shotgun sequencing	A method used for sequencing long DNA strands. It involves breaking up the sample DNA into fragments, determining the sequence of bases in each piece and then using a program to assemble the pieces in order.
Single Nucleotide Polymorphisms (SNPs)	Similar to a variant, polymorphisms are differences in bases at a given position on a DNA sequence. Scientists can study how SNPs in the human genome relate to disease, drug response, and other phenotypes.
SOLiD sequencing	The Applied Biosystems division of Life Technologies Inc. purchased the SOLiD (Supported Oligo Ligation Detection) technology from the biotech company Agencourt Personal Genomics and released the first commercial version of this NGS machine in 2007. The technology is fundamentally different from any other Sanger or NGS method in that it uses ligation of short fluorescently labeled oligonucleotides to a sequencing primer rather than DNA polymerase to copy a DNA template. Sequences are detected 2 bases at a time, and then base calls are made based on two overlapping oligos. Raw data files use a “color space” system that is different from the base calls produced by all other sequencing systems and requires different informatics software. This system has some interesting built-in error correction algorithms but has failed to show superior overall accuracy in the hands of customers. The yield of the system is similar to that of Illumina NGS machines.
Somatic cells	Any cell in the body except egg and sperm cells.
Trait	A specific characteristic, for example eye colour.
Transcription	The first step in making proteins where molecules of RNA are created from DNA. This process happens in the cell’s nucleus.
Translation	The second step in making proteins when an mRNA molecule codes for generating polypeptide chains. This process happens in ribosomes.

Tumour heterogeneity	When a tumour is made up of many different types of cells. This means they have different genes and proteins. For this reason, doctors need to use a combination of treatments to destroy all the different cells.
Tumour Profiling	Finding information about the genes in cancer cells. This usually happens in hospital labs when tumours have been surgically removed and can help doctors decide which treatments may be most effective for a particular cancer.
Variant	DNA bases that are found to be different from a reference genome sequence. This difference may be bases that were deleted, mutated, or new bases that were inserted. We all have lots of different variants. Regardless of whether they arise in the DNA's protein coding regions or not, they don't always lead to diseases. In fact, we struggle to know what they mean. The 100,000 Genomes Project will help uncover information like this.
Variant Call Format (VCF)	A type of text file, similar to what .doc is for a Microsoft Word document. It stores information about all the variant calls, their position in the DNA sequence and can sometimes identify gene names at each position.
Variant calling	The method of finding variants in the sequenced genome compared to a reference genome.
Variant Calls	The results of variant calling i.e. the bases that are different compared to the reference genome.
Variant detection	NGS is frequently used to identify mutations in DNA samples from individual patients or experimental organisms. Sequencing can be done at the whole-genome scale; RNA-seq, which targets expressed genes; exome capture, which targets specific exon regions captured by hybridization to probes of known sequence; or amplicons for genes or regions of interest. In all cases, sequence variants are detected by alignment of NGS reads to a reference sequence and then identification of differences between the reads and the reference. Variant detection algorithms must distinguish between random sequencing errors, differences caused by incorrect alignment, and true variants in the genome of the target organism. Various combinations of base quality scores, alignment quality scores, depth of coverage, variant allele frequency, and the presence of nearby sequence variants and indels are all used to differentiate true variants from false positives. Recent algorithms have also made use of machine learning methods based on training sets of genotype data or large sets of samples from different patients/organisms that are sequenced in parallel with the same sample preparation methods on the same NGS machines.
Variation	Differences in the DNA sequence between individuals and in a population. These do not cause diseases.
Whole Genome Sequencing	Reading along the complete length of DNA, including all exons and introns, to determine the order of every base pair letter by letter.

APPENDIX 2. COMPOSITION OF NGS PANELS

Disclaimer: The BeWG NGS panel composition is the outcome of a first round of expert opinions of Belgian medical professionals on potential markers to be taken up in a cancer gene panel for clinical use. The presence of a marker in this list is to be merely taken at this stage as informative and cannot be considered in whatsoever context as being endorsed by the KCE, the Cancer Centre or the Belgian government for any clinical use.

Table A1. Comparison of five lists with markers/target genes to be potentially included in a NGS gene panel for cancer.

The CAP compared in total 45 cancer gene lists; INCA and BeWG list have been compiled based on national expert opinions; 'Truseq Cancer' represents the 'TruSeq Amplicon - Cancer Panel' (TSACP) from Illumina Inc. while 'Ion Torrent' represents the 'Ion AmpliSeq™ Cancer Hotspot Panel v2' panel from Life Technologies).

Abbreviations: "CAP" = Commission of American Pathologists (US); "INCA" = Institut National de Cancer (France); "BeWG" = Belgian Working group on NGS-TT (this project). 'Illumina' and 'Ion Torrent' stand for commercial panels from the companies Illumina and Life Technologies, respectively).

The number given in the colum CAP is the number of times a particular marker was retained in the respective gene lists collected by CAP based on a survey. The number given under BeWG is the number of times the particants (n=7) indicated the specific marker as having a potential clinical utility.

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent	Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7			# participating labs			n = 7		
ABCC1	1					BAP1	5				
ABL1	18		3	x	x	BARD1	4				
ABL2	2					BCL11B			0		
AFF3	1					BCL2	5				
AKT1	23	x	1	x	x	BCL2L1	2				
AKT2	6					BCL6	4				
AKT3	7					BCOR	5		3		
ALK	27	x	5	x	x	BCR	3		0		
ALOX12B			0			BIRC3		x	1		
APC	27		1	x	x	BLM	4				
AR	4					BMPR1A	9				
ARID1A	4		0			BRAF	25	x	6	x	x
ARID2	3					BRCA1	13				
ASXL1	1	x	4			BRCA2	13				
ATF1	1					BRIP1	9				
ATM	27	x	1	x	x	BTK	2				
ATR	2					CALR			5		
ATRX	3		1			BUB1B	3				
AURKA	1					CARD11	5	x	0		
AXIN2	1					CBFB	1		0		
AXL	3					CBL	8	x	2		

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
CCND1	4		2		
CCND2	3				
CCND3	3		0		
CCNE1	4				
CD70			0		
CD79A	2	x			
CD79B	4	x	0		
CDC73	5				
CD83			0		
CDH1	27		0	x	x
CDH11	1				
CDK12	2				
CDK2	1				
CDK4	8		0		
CDK6	4		0		
CDK8	3				
CDK9	1				
CDKN1B	2				
CDKN2A	22		0	x	x
CDKN2B	3		0		
CEBPA	12	x	3		

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
CHEK1	3				
CHEK2	1				
CIC	2				
CIITA	1				
cKIT	1		4		
CNOT			0		
COL1A1	1				
CREB1	1				
CREBBP	7		0		
CRKL	3				
CRLF2	4				
CRTC1	2				
CSF3R			4		
CSF1R	14		0	x	x
CSMD3	1				
CTLA-4			0		
CTCF	1				
CTNNA1	3				
CTNNB1	2		1	x	x
CUX1			1		
CYLD	4				

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent	Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7			# participating labs			n = 7		
CXCR4			0			EPHA7	2				
DAXX	2					EPHB1	2				
DDB2	3					EPOR			2		
DDIT3	1					ERBB2	2	x	3	x	x
DDR2	8	x				ERBB3	4				
DDX3X			0			ERBB4	15		1	x	x
DEK	1		0			ERCC1	1				
DICER1	3					ERCC2	4				
DIS3			0			ERCC3	3				
DMD	1					ERCC4	3				
DNMT3A	9	x	2			ERCC5	3				
DPYD	2		0			ERG	2				
EBF1			0			ESR1	5		0		
EED			0			ETV1	3				
EGFR	24	x	4	x	x	ETV4	3				
EGLN (PHD2)			2			ETV5	2				
EP300	5		0			ETV6	6	x	2		
EPAS1 (HIF2A)			2			EWSR1	2				
EPCAM	13					EXT1	3				
EPHA3	4					EXT2	3				
EPHA5	3					EZH2	15	x	2		x

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent	Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7			# participating labs			n = 7		
CXCR4			0			EPHA7	2				
DAXX	2					EPHB1	2				
DDB2	3					EPOR			2		
DDIT3	1					ERBB2	2	x	3	x	x
DDR2	8	x				ERBB3	4				
DDX3X			0			ERBB4	15		1	x	x
DEK	1		0			ERCC1	1				
DICER1	3					ERCC2	4				
DIS3			0			ERCC3	3				
DMD	1					ERCC4	3				
DNMT3A	9	x	2			ERCC5	3				
DPYD	2		0			ERG	2				
EBF1			0			ESR1	5		0		
EED			0			ETV1	3				
EGFR	24	x	4	x	x	ETV4	3				
EGLN (PHD2)			2			ETV5	2				
EP300	5		0			ETV6	6	x	2		
EPAS1 (HIF2A)			2			EWSR1	2				
EPCAM	13					EXT1	3				
EPHA3	4					EXT2	3				
EPHA5	3					EZH2	15	x	2		x

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
FAM46C			0		
FAM123B	2				
FANCA	4				
FANCC	5				
FANCD2	5				
FANCE	3				
FANCF	4				
FANCG	4				
FAS	2				
FBXW7	18	x	1	x	x
FGFR1	19		1	x	x
FGFR2	19	x	1	x	x
FGFR3	17		0	x	x
FGFR4	7				
FH	4				
FLCN	4				
FLI1	1				
FLT1	5				
FLT3	15	x	4	x	x
FLT4	5				
FOXL2	4				

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
FOXO1	1				
FOXP1	1				
GATA1	6		2		
GATA2	8		0		
GATA3	3		0		
GATA4	1				
GLI1	1				
GNA11	17		1	x	x
GNAQ	18		1	x	x
GNAS	18		1	x	x
GPR124	2				
GREM1	1				
GSTM5	1				
HER2			4		
HER2/neu			4		
HLA-A			0		
HNF1A	14		0	x	x
HOOK3	1				
HOX1			1		
HOXB13	1				
HRAS	22	x	1	x	x

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
IDH1	22	x	4	x	x
IDH2	2	x	4		x
IGF1R	4				
IGF2R	2				
IKBKE	2				
IKZF1	5		2		
IKZF2/3			0		
IL7R	6		0		
INHBA	1				
IRF4	2				
IRS2	2				
ITGB3	1				
JAK1	5		1		
JAK2	18	x	5	x	x
JAK3	16	x	6	x	x
JUN	2				
KDM5C	2				
KDM6A	6		1		
KDR	15		0	x	x
KEAP1	2				
KIT	23	x	5	x	x

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
KLF6	1				
KRAS	26	x	6	x	x
LEF1			0		
LMO1	1				
LMO2	1				
LMO3	1				
LNK			2		
LRP1B	2				
LYL1			0		
LTK	1				
MAGED1			0		
MAML2	1				
MAP2K1	12				
MAP2K2	4				
MAP2K4	5				
MAP3K1	2				
MAP3K7	1				
MAPK1	4		1		
MCL1	4				
MDM2	4				
MDM4	4				

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
MEF2B			0		
MEN1	8				
MEK			1		
MET	22	x	3	x	x
MITF	5				
MLH1	27			x	x
MLH3	1				
MLL	6		2		
MLL2	3		1		
MLL3	1		1		
MPL	19	x	4	x	x
MRE11A	6				
MSH2	18				
MSH6	19				
MTHFR	1				
MTOR	4		1		
MUTYH	13				
MYB	2				
MYC	6				
MYCL1	4				
MYCN	4				

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
MYD88	6	x	4		
MYH11			1		
NBN	8				
NCOA2	1				
NF1	12		0		
NF2	6				
NFE2L2	3				
NFKB1	1				
NFKB2	1				
NFKBIZ	1				
NKX2-1	4				
NOTCH1	21	x	2	x	x
NOTCH2	6	x			
NPM1	19	x	3	x	x
NRAS	25	x	6	x	x
NTRK1	4				
NTRK2	4				
NTRK3	5				
NUP214			1		
P2RX5			0		
P2RY8			0		

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
PAK3	2				
PALB2	11				
PARP1	2		1		
PAX3	1				
PAX5	7		1		
PAX8	1				
PBRM1	3				
PDE4DIP	1				
PDGFRA	2	x	3	x	x
PDGFRB	5		2		
PHF6	2		3		
PHOX2B	3				
PIK3C2A	1				
PIK3C2B	2				
PIK3CA	24	x	2	x	x
PIK3CB	1				
PIK3CD	1				
PIK3CG	2				
PIK3R1	8				
PIK3R2	3				
PIK3R5	1				

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
PIM1	2				
PKHD1	1				
PML			1		
PMS1	4				
PMS2	16				
POT1			0		
PPP2R1A	3				
PRDM1	4				
PRKAR1A	5				
PRKDC	3				
PTCH1	8				
PTEN	36	x	2	x	x
PTGS2	2				
PTPN11	18	x	2	x	x
PTPRD	3				
RAC1	1				
RAD21			0		
RAD50	5				
RAD51C	6				
RAD51D	5				
RAF1	4				

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
RARA	4		1		
RB1	17			x	x
REL	2				
RET	21		1	x	x
RICTOR	2				
RNASEL	1				
RON		x			
ROS1	6		1		
RPL5/10			0		
RRM1	1				
RUNX1	12	x	3		
RUNX1T1	1		1		
SBDS	3				
SDHA	4				
SDHB	6				
SDHD	6				
SETBP1	1		3		
SETD2	4		0		
SF3B1	7	x	4		
SMAD2	5				
SMAD4	22		0	x	x

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
SMARCA4	5				
SMC3			1		
SMARCB1	15		0	x	x
SMO	16		0	x	x
SOCS1	4				
SRC	14		0	x	x
SFRS1			1		
SRSF2	5	x	3		
SSX1	1				
STAG2	2		2		
STK11	33	x	0	x	x
SUFU	4				
SUZ12	3		0		
SYK	2				
SYNE1	1				
TAL1			1		
TCF3	2				
TCF7L1	2				
TCF7L2	2				
TERT	3				
TET2	9	x	5		

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
TFE3	1		4		
TGFBR2	4				
THBS1	1				
TLR2			0		
TLR4	1				
TNFAIP3	5	s			
TNFRSF14	2				
TNK2	1				
TOP1	3				
TP53	39	x	4	x	x
TRRAP	3				
TSC1	7				
TSC2	7				
TSHR	3				
U2AF1	3	x	3		

Targets	CAP	INCA	BeWG	Illumina	Ion Torrent
# participating labs			n = 7		
U2AF2			2		
UTX/KDM6A			0		
UGT1A1	2				
VEGF			1		
VEGFR2			0		
VHL	22		3	x	x
WHSC1			0		
WHSC1L1			0		
WRN	3				
WT1	11		4		
XPA	3				
XPC	3				
XPO1	5		0		
XRCC2	2				
ZRSR2	5		4		

Table A2. Outcome of the survey amongst the participants of the project: cancer markers which are potentially clinically useful, their type of clinical utility and the technologies applied for their detection.

Myeloid, lymphoid and solid lists were compiled beforehand by the working group. Illumina and Ion torrent panels are listed as described in the commercial brochures of the respective companies. 'Truseq Cancer' represents the 'TruSeq Amplicon - Cancer Panel' (TSACP) from Illumina Inc. while 'Ion Torrent' represents the 'Ion AmpliSeq™ Cancer Hotspot Panel v2' panel from Life Technologies).

A colour index was introduced to visualize the number of labs scoring a particular combination useful; two types of combinations are evaluated: 'target x clinical utility' or 'target x practice'. Colour code: 'gray' = less then 3 participants considered a combination relevant; 'green' = three or more participants considered this combination relevant. The numbers indicated in the table corresponds to the number of participants scoring a particular combination as useful.

A 'maxCU' (maximal commonly estimated clinical utility for a particular use) was set as the highest score for clinical use of a particular target by the participants.

All targets which have obtained a 'maxCU' score ≥ 3 are indicated in a green box.

NGS GENE PANEL: candidate genes, target description and clinical utility

Target genes	Myeloid (BeWG)	Lymloid (BeWG)	Solid (Be WG)	Illumina	Ion Torrent	Score	Clinical Utility: Predispositon	diagnostic	prognostic	predictive	Follow up	Current Practice: NGS	Cytogenetics	FISH	PCR	Max CU
BRAF	1	1	1	1	1	5	0	3	4	6	1	5	0	0	4	6
CDKN2A	1	1	1	1	1	5	0	0	0	0	0	2	0	0	0	0
KRAS	1	1	1	1	1	5	0	1	2	6	0	5	0	0	4	6
NRAS	1	1	1	1	1	5	0	1	3	6	0	5	0	0	4	6
PTEN	1	1	1	1	1	5	0	1	1	2	0	4	0	0	2	2
TP53	1	1	1	1	1	5	0	1	4	1	0	3	0	0	3	4
ABL1	1		1	1	1	4	0	1	0	3	0	4	0	0	3	3
FBXW7	1	1		1	1	4	0	0	1	1	0	3	0	0	1	1
FLT3	1	1		1	1	4	0	2	4	1	1	4	0	0	6	4
NOTCH1		1	1	1	1	4	0	0	2	0	0	3	0	0	2	2
PTPN11	1	1		1	1	4	0	2	1	0	0	3	0	0	1	2
AKT1			1	1	1	3	0	0	1	1	0	2	0	0	0	1
ALK			1	1	1	3	0	1	1	5	0	1	0	4	1	5
ATM		1		1	1	3	0	0	1	1	0	1	0	1	0	1
CDH1			1	1	1	3	1	0	0	0	0	1	0	0	0	1
EGFR			1	1	1	3	0	0	0	4	0	4	0	1	4	4
ERBB2	1			1	1	3	0	1	2	3	0	3	0	3	0	3
ERBB4	1			1	1	3	0	0	0	1	0	0	0	0	0	1
EZH2	1	1			1	3	0	2	2	1	0	4	0	0	3	2
FGFR1			1	1	1	3	0	0	0	1	0	0	0	1	0	1
FGFR2			1	1	1	3	0	0	0	1	0	2	0	1	0	1
GNAS	1			1	1	3	0	1	0	1	0	2	0	0	0	1
HRAS	1			1	1	3	0	0	1	1	0	3	0	0	1	1
IDH1	1			1	1	3	0	2	4	1	0	4	0	0	3	4
JAK2	1			1	1	3	1	5	1	1	1	4	0	0	6	5
JAK3	1			1	1	3	0	0	0	0	0	3	0	0	1	0
KIT	1			1	1	3	0	4	2	5	0	4	0	0	4	5
MET			1	1	1	3	0	1	1	3	0	2	0	1	0	3
MPL	1			1	1	3	0	4	0	1	0	4	0	0	4	4
NPM1	1			1	1	3	0	2	3	1	1	4	0	0	6	3
PDGFRA	1			1	1	3	0	3	1	3	1	4	0	2	4	3

NGS GENE PANEL: candidate genes, target description and clinical utility

Target genes	Myeloid (BeWG)	Lymloid (BeWG)	Solid (Be WG)	Illumina	Ion Torrent	Score	Clinical Utility: Predispositon	diagnostic	prognostic	predictive	Follow up	Current Practice: NGS	Cytogenetics	FISH	PCR	Max CU
BRAF	1	1	1	1	1	5	1	1	0	1	0	2	0	0	0	1
CDKN2A	1	1	1	1	1	5	0	0	0	0	0	0	0	0	0	0
KRAS	1	1	1	1	1	5	0	2	0	0	0	0	0	1	1	2
NRAS	1	1	1	1	1	5	0	0	0	0	0	0	0	0	0	0
PTEN	1	1	1	1	1	5	1	1	0	1	0	2	0	0	0	1
TP53	1	1	1	1	1	5	0	1	4	1	0	3	0	0	3	4
ABL1	1		1	1	1	4	0	1	2	1	0	4	0	0	2	2
FBXW7	1	1		1	1	4	0	2	2	0	0	3	0	0	2	2
FLT3	1	1		1	1	4	0	0	0	0	0	0	0	0	0	0
NOTCH1		1	1	1	1	4	0	0	0	1	0	1	0	0	1	1
PTPN11	1	1		1	1	4	0	0	0	1	0	2	0	0	1	1
AKT1			1	1	1	3	0	0	0	0	0	0	0	0	0	0
ALK			1	1	1	3	0	3	4	1	0	4	0	0	4	4
ATM		1		1	1	3	0	0	0	0	0	0	0	0	0	0
CDH1			1	1	1	3	0	2	0	0	0	1	0	0	2	2
EGFR			1	1	1	3	1	0	1	0	0	0	0	0	0	1
ERBB2	1			1	1	3	0	2	3	0	0	3	0	0	2	3
ERBB4	1			1	1	3	0	1	0	0	0	0	0	1	0	1
EZH2	1	1			1	3	0	2	3	1	0	4	0	0	2	3
FGFR1			1	1	1	3	0	4	3	1	0	4	0	0	3	4
FGFR2			1	1	1	3	0	0	0	0	0	2	0	0	0	0
GNAS	1			1	1	3	0	0	0	0	0	0	0	0	0	0
HRAS	1			1	1	3	0	0	0	0	0	1	0	0	0	0
IDH1	1			1	1	3	0	0	0	0	0	2	0	0	0	0
JAK2	1			1	1	3	0	0	0	0	0	0	0	0	0	0
JAK3	1			1	1	3	0	4	3	1	1	4	0	0	2	4
KIT	1			1	1	3	0	0	0	0	0	0	0	0	0	0
MET			1	1	1	3	0	2	1	0	1	0	0	2	0	2
MPL	1			1	1	3	0	4	0	1	0	4	0	0	4	4
NPM1	1			1	1	3	0	0	0	0	0	0	0	0	0	0
PDGFRA	1			1	1	3	0	0	0	0	0	0	0	0	0	0
suz12	1	1				2	0	0	0	0	0	0	0	0	0	0
WT1	1	1				2	0	4	3	1	1	4	0	0	2	4
SMO			1			1	0	0	0	0	0	0	0	0	0	0
14q32		1				1	0	2	1	0	1	0	0	2	0	2

NGS GENE PANEL: candidate genes, target description and clinical utility

Target genes	Myeloid (BeWG)	Lymloid (BeWG)	Solid (Be WG)	Illumina	Ion Torrent	Score	Clinical Utility: Predisposiiton	diagnostic	prognostic	predictive	Follow up	Current Practice: NGS	Cytogenetics	FISH	PCR
ALOX12B		1				*1*	0	0	0	0	0	0	0	0	0
ARID1A		1				*1*	0	0	0	0	0	0	0	0	0
ASXL1	1					*1*	0	2	4	1	0	4	0	0	3	4
ATRX	1					*1*	0	0	1	0	0	2	0	0	0	1
BCL11B		1				*1*	0	0	0	0	0	1	0	0	0	0
BCOR/BCORL1	1					*1*	0	1	3	1	0	3	0	0	2	3
BIRC3		1				*1*	0	0	1	0	0	1	0	0	1	1
BRAF2 (?)			1			*1*	0	0	0	0	0	0	0	0	0	0
CALR	1					*1*	0	5	1	1	0	3	0	0	5	5
CARD11		1				*1*	0	0	0	0	0	0	0	0	0	0
CBFB	1					*1*	0	0	1	0	0	1	0	0	0	1
CBL	1					*1*	0	2	2	1	0	3	0	0	2	2
CBL B/C	1					*1*	0	0	0	0	0	2	0	0	0	0
CCND3		1				*1*	0	0	0	0	0	0	0	0	0	0
CD70		1				*1*	0	0	0	0	0	0	0	0	0	0
CD79B		1				*1*	0	0	0	0	0	0	0	0	0	0
CD83		1				*1*	0	0	0	0	0	0	0	0	0	0
CDK4			1			*1*	0	0	0	0	0	0	0	0	0	0
CDK6			1			*1*	0	0	0	0	0	0	0	0	0	0
CDKN2B		1				*1*	0	0	0	0	0	0	0	0	0	0
CEBPA	1					*1*	0	3	3	1	0	4	0	0	3	3
c-KIT			1			*1*	0	2	1	4	0	4	0	0	3	4
CNOT		1				*1*	0	0	0	0	0	0	0	0	0	0
CREBBP		1				*1*	0	0	0	0	0	0	0	0	0	0
CSF3R	1					*1*	1	4	2	2	0	3	0	0	2	4
CTLA-4			1			*1*	0	0	0	0	0	0	0	0	0	0
CUX1	1					*1*	0	1	1	0	0	3	0	0	0	1
CXCR4		1				*1*	0	0	0	0	0	0	0	0	0	0
DDX3X		1				*1*	0	0	0	0	0	0	0	0	0	0
DEK	1					*1*	0	1	1	0	0	0	0	1	0	1
del(11q22)		1				*1*	0	0	3	0	0	0	0	3	1	3
del(17p13)		1				*1*	0	0	3	0	0	0	0	3	1	3
del(X)		1				*1*	0	0	0	0	0	0	0	1	1	0
del13q14		1				*1*	0	1	2	0	0	0	0	3	1	2
DIS3		1				*1*	0	0	0	0	0	0	0	0	0	0
DPYD			1			*1*	0	0	0	0	0	0	0	0	0	0

NGS GENE PANEL: candidate genes, target description and clinical utility

Target genes	Myeloid (BeWG)	Lymloid (BeWG)	Solid (Be WG)	Illumina	Ion Torrent	Score	Clinical Utility: Predispositon	diagnostic	prognostic	predictive	Follow up	Current Practice: NGS	Cytogenetics	FISH	PCR
EBF1		1				1	0	0	0	0	0	0	0	0	0
EED		1				1	0	0	0	0	0	0	0	0	0
EGLN (PHD2)	1					1	1	2	0	0	1	0	0	2	2
EP300		1				1	0	0	0	0	1	0	0	0	0
EPAS1 (HIF2A)	1					1	1	2	0	0	1	0	0	2	2
EPOR	1					1	1	2	0	0	1	0	0	2	2
ESR1			1			1	0	0	0	0	0	0	0	0	0
FAM46C		1				1	0	0	0	0	0	0	0	0	0
GATA1	1					1	0	2	0	1	0	3	0	0	1	2
GATA2	1					1	0	0	0	0	0	3	0	0	1	0
GATA3		1				1	0	0	0	0	0	0	0	0	0	0
HER2			1			1	0	1	0	4	0	2	0	4	1	4
HER2/neu			1			1	0	1	0	4	0	2	0	3	1	4
HLA-A		1				1	0	0	0	0	0	0	0	0	0	0
HOX1		1				1	0	0	1	0	0	0	0	0	1	1
IKZF1	1					1	0	0	2	0	0	3	0	0	1	2
IKZF1		1				1	0	0	1	0	0	2	0	0	0	1
IKZF2/3		1				1	0	0	0	0	0	0	0	0	0	0
IL7R		1				1	0	0	0	0	0	1	0	0	1	0
JAK1		1				1	0	0	1	0	0	1	0	0	1	1
JAK3		1				1	0	0	2	0	0	3	0	0	1	2
KDM6A	1					1	0	0	1	0	0	2	0	0	0	1
LEF1		1				1	0	0	0	0	0	0	0	0	0	0
LYL1		1				1	0	0	0	0	0	0	0	0	0	0
MAGED1		1				1	0	0	0	0	0	0	0	0	0	0
MAPK1		1				1	0	0	1	0	0	1	0	0	0	1
MEF2B		1				1	0	0	0	0	0	0	0	0	0	0
MEK			1			1	0	0	1	1	0	1	0	0	0	1
MLL2/3		1				1	0	0	1	0	0	2	0	0	0	1
MLL-PTD	1					1	0	1	2	0	0	2	0	2	2	2
mTOR			1			1	0	0	1	1	0	0	0	0	0	1
MYH11	1					1	0	1	0	0	0	0	0	1	1	1
NF1		1				1	0	0	0	0	0	0	0	0	0	0
NUP214	1					1	0	1	1	1	0	0	0	1	1	1
NUP214-ABL1		1				1	0	1	1	1	0	0	0	2	1	1

NGS GENE PANEL: candidate genes, target description and clinical utility

Target genes	Myeloid (BeWG)	Lymloid (BeWG)	Solid (Be WG)	Illumina	Ion Torrent	Score	Clinical Utility: Predispositon	Clinical Utility: diagnostic	Clinical Utility: prognostic	Clinical Utility: predictive	Clinical Utility: Follow up	Current Practice: NGS	Current Practice: Cytogenetics	Current Practice: FISH	Current Practice: PCR
P2RX5		1				1	0	0	0	0	0	0	0	0	0
P2RY8		1				1	0	0	0	0	0	0	0	0	0
p53(germline)		1				1	0	0	3	0	0	2	0	0	1	3
PARP1			1			1	0	0	0	1	0	0	0	0	0	1
PAX5		1				1	0	1	0	0	0	0	0	0	0	1
PDGFR			1			1	0	1	1	2	0	2	0	1	1	2
PDGFRB	1					1	0	2	1	1	0	1	0	2	2	2
PML	1					1	0	1	1	1	0	0	0	1	1	1
POT1		1				1	0	0	0	0	0	0	0	0	0	0
RAD21	1					1	0	0	0	0	0	1	0	0	0	0
Rank ligand			1			1	0	0	0	0	0	0	0	0	0	0
RARA	1					1	0	1	1	1	0	0	0	2	2	1
ROS			1			1	0	0	0	1	0	0	0	2	0	1
RPL5/10		1				1	0	0	0	0	0	0	0	0	0	0
RUNX1T1	1					1	0	1	1	1	0	1	0	1	1	1
SETBP1	1					1	0	3	1	0	0	3	0	0	3	3
SETD2		1				1	0	0	0	0	0	0	0	0	0	0
SFRS1		1				1	0	1	1	0	0	0	0	0	1	1
SMC3	1					1	0	1	1	0	0	2	0	0	1	1
Smoothened receptor			1			1	0	0	0	0	0	0	0	0	0	0
SRSF2	1					1	0	2	3	0	0	3	0	0	2	3
STAG2	1					1	0	1	2	0	0	3	0	0	1	2
t(1;19)		1				1	0	3	2	0	2	0	1	2	4	3
t(11q23)		1				1	0	3	2	0	1	0	1	4	2	3
t(12;21)		1				1	0	3	2	0	2	0	1	2	4	3
t(9;22)		1				1	0	3	2	2	2	0	1	2	4	3
t(X;14)		1				1	0	2	0	0	1	0	0	1	2	2
TAL1		1				1	0	1	1	0	0	0	0	1	1	1
TET2	1					1	0	2	5	0	0	4	0	0	2	5
TLR2		1				1	0	0	0	0	0	0	0	0	0	0
trisomie 12		1				1	0	1	2	0	0	0	1	4	1	2
U2AF1	1					1	0	1	3	0	0	2	0	0	1	3

NGS GENE PANEL: candidate genes, target description and clinical utility

Target genes	Myeloid (BeWG)	Lymloid (BeWG)	Solid (Be WG)	Illumina	Ion Torrent	Score	Clinical Utility: Predispositon	diagnostic	prognostic	predictive	Follow up	Current Practice: NGS	Cytogenetics	FISH	PCR
U2AF2		1				1	0	2	1	0	0	0	0	0	2	2
UTX/KDM6A		1				1	0	0	0	0	0	0	0	0	0	0
VEGF			1			1	0	0	1	1	0	0	0	0	0	1
VEGFR2			1			1	0	0	0	0	0	0	0	0	0	0
WHSC1		1				1	0	0	0	0	0	0	0	0	0	0
WHSC1L1		1				1	0	0	0	0	0	0	0	0	0	0
XPO1		1				1	0	0	0	0	0	0	0	0	0	0
ZRSR2	1					1	0	2	4	0	0	3	0	0	2	4
FGFR1 fusions	1					1	0	3	2	1	0	0	1	2	1	3
fusion CBFB-MYH11	1					1	0	5	3	1	2	0	3	3	5	5
MLL-fusions	1					1	0	5	3	0	1	0	3	5	3	5
PDGFRA fusions	1					1	0	4	1	1	1	0	1	3	3	4
fusion BCR-ABL	1		1			2	0	5	4	4	4	0	3	3	6	5
fusion PML-RARA	1					1	0	5	4	4	4	0	3	3	5	5
fusion RUNX1-RUNXT1	1					1	0	5	4	1	3	0	3	3	5	5
fusion CBFB-MYH11	1					1	0	5	4	1	3	0	3	3	5	5
fusion DEK-NUP214	1					1	0	4	4	1	2	0	3	1	3	4
fusions to PDGFRB		1				1	0	4	3	1	2	0	2	4	1	4
fusions to ABL1		1				1	0	3	0	1	2	0	2	1	1	3
fusions to JAK2		1				1	0	3	1	1	2	0	1	1	1	3

COLOR Code	
	Myeloid
	Lymloid
	Solid
	Illumina Truseq 48 genes
	Ion Torrent

CU ndicated by participants as relevant	
	< 3 participants
	≥3 participants

Some selected references on the clinical utility for hemato-oncology genes (no systematic review was undertaken).

General	http://www.ncbi.nlm.nih.gov/pubmed/24106950
ASXL1	http://www.ncbi.nlm.nih.gov/pubmed/19388938 http://www.ncbi.nlm.nih.gov/pubmed/20182461
BCOR/BCORL1	http://www.ncbi.nlm.nih.gov/pubmed/24047651 http://www.ncbi.nlm.nih.gov/pubmed/21989985 http://www.ncbi.nlm.nih.gov/pubmed/22012066
CALR	http://www.ncbi.nlm.nih.gov/pubmed/24325356 http://www.ncbi.nlm.nih.gov/pubmed/24325359
CBL	http://www.ncbi.nlm.nih.gov/pubmed/19620960
CSF3R	http://www.ncbi.nlm.nih.gov/pubmed/23656643
CUX1	http://www.ncbi.nlm.nih.gov/pubmed/24316979
DNMT3A	http://www.ncbi.nlm.nih.gov/pubmed/21067377
EZH2	http://www.ncbi.nlm.nih.gov/pubmed/20601954
FLT3	http://www.ncbi.nlm.nih.gov/pubmed/22417203
IDH1	http://www.ncbi.nlm.nih.gov/pubmed/19657110
JAK2	http://www.ncbi.nlm.nih.gov/pubmed/15793561 http://www.ncbi.nlm.nih.gov/pubmed/15858187 http://www.ncbi.nlm.nih.gov/pubmed/15837627
MPL	http://www.ncbi.nlm.nih.gov/pubmed/16834459
NPM1	http://www.ncbi.nlm.nih.gov/pubmed/15659725
PTPN11	http://www.ncbi.nlm.nih.gov/pubmed/14982869
RUNX1	http://www.ncbi.nlm.nih.gov/pubmed/12393381
SF3B1	http://www.ncbi.nlm.nih.gov/pubmed/21995386
TET2	http://www.ncbi.nlm.nih.gov/pubmed/19483684

APPENDIX 3. SEARCH STRATEGIES - IMPACT OF TEST ACCURACY ON ICERS

Appendix 3.1. PUBMED

("Neoplasms"[Mesh]) AND (((((((("Sequence Analysis, DNA"[Mesh]) OR ((DNA) OR gene)) OR ((sequence) OR sequencing)) OR ((companion) AND diagnos*)) OR ((molecular) AND diagnos*))) AND ((((((((("Economics"[Mesh] OR "Economics, Pharmaceutical"[Mesh] OR "Economics, Medical"[Mesh] OR "Economics, Nursing"[Mesh] OR "Economics, Hospital"[Mesh] OR "Health Care Economics and Organizations"[Mesh])) OR ("Costs and Cost Analysis"[Mesh])) OR "Quality-Adjusted Life Years"[Mesh]) OR "Value of Life"[Mesh]) OR budget*[Title/Abstract]) OR ((price[Title/Abstract]) OR pricing[Title/Abstract])) OR ((cost effectiveness[Title/Abstract]) OR cost-effectiveness[Title/Abstract])) OR ((cost-utility[Title/Abstract]) OR cost utility[Title/Abstract])) OR ((cost benefit[Title/Abstract]) OR cost-benefit[Title/Abstract]))) AND ("Sensitivity and Specificity"[Mesh])) AND ("2010/01/01"[PDat] : "2014/12/31"[PDat]))

Appendix 3.2. EMBASE

No. Query Results Results Date:04/Aug/2014

#36. 'sensitivity and specificity'/exp AND 'neoplasm'/exp AND [embase]/lim AND ('dna sequence'/exp AND [embase]/lim OR (gene:ab,ti OR dna:ab,ti AND [embase]/lim) OR (molecular AND diagnos* AND [embase]/lim) OR (companion AND diagnos* AND [embase]/lim) OR (sequencing:ab,ti OR sequence:ab,ti AND [embase]/lim)) AND ('economics'/exp AND [embase]/lim OR ('health care cost'/exp AND [embase]/lim) OR ('health care financing'/exp AND [embase]/lim) OR ('quality adjusted life year'/exp AND [embase]/lim) OR ('cost effectiveness analysis'/exp AND [embase]/lim) OR ('cost utility analysis'/exp AND [embase]/lim) OR ('cost benefit analysis'/exp AND [embase]/lim) OR ('cost minimization analysis'/exp AND [embase]/lim) OR ('economic evaluation'/exp AND [embase]/lim)) AND (2005:py OR 2006:py OR 2007:py OR 2008:py OR 2009:py OR 2010:py OR 2011:py OR 2012:py OR 2013:py OR 2014:py) — 294

#35. 'sensitivity and specificity'/exp AND 'neoplasm'/exp AND [embase]/lim AND ('dna sequence'/exp AND [embase]/lim OR (gene:ab,ti OR dna:ab,ti AND [embase]/lim) OR (molecular AND diagnos* AND [embase]/lim) OR (companion AND diagnos* AND [embase]/lim) OR (sequencing:ab,ti OR sequence:ab,ti AND [embase]/lim)) AND ('economics'/exp AND [embase]/lim OR ('health care cost'/exp AND [embase]/lim) OR ('health care financing'/exp AND [embase]/lim) OR ('quality adjusted life year'/exp AND [embase]/lim) OR ('cost effectiveness analysis'/exp AND [embase]/lim) OR ('cost utility analysis'/exp AND [embase]/lim) OR ('cost benefit analysis'/exp AND [embase]/lim) OR ('cost minimization — 349

analysis'/exp AND [embase]/lim) OR ('economic evaluation'/exp AND [embase]/lim))	
#34. 'economics'/exp AND [embase]/lim OR ('health care cost'/exp AND [embase]/lim) OR ('health care financing'/exp AND [embase]/lim) OR ('quality adjusted life year'/exp AND [embase]/lim) OR ('cost effectiveness analysis'/exp AND [embase]/lim) OR ('cost utility analysis'/exp AND [embase]/lim) OR ('cost benefit analysis'/exp AND [embase]/lim) OR ('cost minimization analysis'/exp AND [embase]/lim) OR ('economic evaluation'/exp AND [embase]/lim)	304,694
#33. 'dna sequence'/exp AND [embase]/lim OR (gene:ab,ti OR dna:ab,ti AND [embase]/lim) OR (molecular AND diagnos* AND [embase]/lim) OR (companion AND diagnos* AND [embase]/lim) OR (sequencing:ab,ti OR sequence:ab,ti AND [embase]/lim)	2,069,480
#32. 'neoplasm'/exp AND [embase]/lim	2,625,961
#27. sequencing:ab,ti OR sequence:ab,ti AND [embase]/lim	581,225
#24. 'economic evaluation'/exp AND [embase]/lim	169,317
#19. 'cost minimization analysis'/exp AND [embase]/lim	2,496
#18. 'cost benefit analysis'/exp AND [embase]/lim	44,127
#17. 'cost utility analysis'/exp AND [embase]/lim	5,538
#16. 'cost effectiveness analysis'/exp AND [embase]/lim	98,749
#15. 'sensitivity and specificity'/exp AND [embase]/lim	137,578
#14. 'quality adjusted life year'/exp AND [embase]/lim	11,198
#11. 'health care financing'/exp AND [embase]/lim	11,316
#9. 'health care cost'/exp AND [embase]/lim	171,817
#8. 'economics'/exp AND [embase]/lim	17,455
#7. companion AND diagnos* AND [embase]/lim	2,444
#6. molecular AND diagnos* AND [embase]/lim	217,052
#2. gene:ab,ti OR dna:ab,ti AND [embase]/lim	1,633,486
#1. 'dna sequence'/exp AND [embase]/lim	117,534

Appendix 3.3. EconLit

Search Strategy:

<1886 to July 2014>

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1 (sequence or sequencing).mp. [mp=heading words, abstract, title, country as subject] (4014)

2 (DNA or gene or genetic).mp. [mp=heading words, abstract, title, country as subject] (2505)

3 (molecular and diagnos*).mp. [mp=heading words, abstract, title, country as subject] (7)

4 (companion and diagnos*).mp. [mp=heading words, abstract, title, country as subject] (4)

5 (neoplasm* or cancer).mp. [mp=heading words, abstract, title, country as subject] (1008)

6 (sensitivity or specificity).mp. [mp=heading words, abstract, title, country as subject] (9226)

7 1 or 2 or 3 or 4 (6456)

8 5 and 6 and 7 (2)

Appendix 3.4. CRD NHS databases

MeSH DESCRIPTOR Sequence Analysis, DNA EXPLODE ALL TREES	86	
2	MeSH DESCRIPTOR Neoplasms EXPLODE ALL TREES IN NHSEED,HTA	5088
3	(companion) AND (diagnos*) IN NHSEED, HTA	22
4	(DNA) OR (gene) IN NHSEED, HTA	643
5	(sequence) OR (sequencing) IN NHSEED, HTA	337
6	(cost*) IN NHSEED, HTA	18750
7	#1 OR #3 OR #4 OR #5	864
8	#2 AND #6 AND #7	192
9	(sensitivity) OR (specificity) IN NHSEED, HTA	9712
10	#8 AND #9	139

APPENDIX 4. MOLECULAR TESTS BY PATHOLOGY, WITH BILLING CODES

Gene	Aberration type	Pathology	Phase	Sample types	DNA or RNA or nuclei	Techniques	Time impl.	Nomenclature billing codes	Max	Reimb. in practice	DNA-NGS possible?	Why not NGS?
Chimerism post-allo-Tx	chimerism	AL	Follow-up	BM	DNA or nuclei	PCR or FISH	0	588814-588825	1	Yes	No	Distinct indication
ALK / 2p23	translocation	ALCL	Diagnosis	FFPE, FT	nuclei	FISH	0	588453-588464	3	Yes	No	Pathology-specific aberration
MLL	translocation	ALL	Diagnosis	B, BM	RNA or nuclei	PCR or FISH	0	588431-588442	5	No	No	RNA analysis
BCR-ABL1 / t(9;22)(q34;q11)	translocation	ALL	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588442	5	Yes	No	RNA analysis
TCF3-PBX / t(1;19)(q23;p13)	translocation	ALL	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588444	5	Yes	No	RNA analysis
BCR-ABL1 / t(9;22)(q34;q11)	translocation	ALL	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588444	5	Yes	No	RNA analysis
ETV6-RUNX1 /	translocation	ALL	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588445	5	Yes	No	RNA analysis
MLL-AFF1 / t(4;11)(q21;q23)	translocation	ALL	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588445	5	Yes	No	RNA analysis
BCR-ABL1 / t(9;22)(q34;q11)	translocation	ALL	Follow-up	B, BM	RNA	RT-PCR	0	588571-588582	1	Yes	No	Individual specific follow-up marker
TCF3-PBX / t(1;19)(q23;p13)	translocation	ALL	Follow-up	B, BM	RNA	RT-PCR	0	588571-588584	1	Yes	No	Individual specific follow-up marker
BCR-ABL1 / t(9;22)(q34;q11)	translocation	ALL	Follow-up	B, BM	RNA	RT-PCR	0	588571-588584	1	Yes	No	Individual specific follow-up marker
ETV6-RUNX1 / t(12;21)(p13;q22)	translocation	ALL	Follow-up	B, BM	RNA	RT-PCR	0	588571-588585	1	Yes	No	Individual specific follow-up marker
MLL-AFF1 / t(4;11)(q21;q23)	translocation	ALL	Follow-up	B, BM	RNA	RT-PCR	0	588571-588585	1	Yes	No	Individual specific follow-up marker
MLL	translocation	AML	Diagnosis	B, BM	RNA or nuclei	PCR or FISH	0	588431-588442	5	No	No	RNA analysis
RUNX1-RUNX1T1 /	translocation	AML	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588442	5	Yes	No	RNA analysis
CBFB-MYH11 / inv(16)(p13;q22)	translocation	AML	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588443	5	Yes	No	RNA analysis
PML-RARA / t(15;17)(q22;q12)	translocation	AML	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588443	5	Yes	No	RNA analysis
BCR-ABL1 / t(9;22)(q34;q11)	translocation	AML	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588444	5	Yes	No	RNA analysis
DEK-NUP214 / t(6;9)(p23;q34)	translocation	AML	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588431-588444	5	Yes	No	RNA analysis
NPM1	mutation	AML	Diagnosis	B, BM	DNA	PCR or Sanger	2	588431-588442	5	No	Yes	
FLT3-ITD	mutation	AML	Diagnosis	B, BM	DNA	PCR or Sanger	2	588431-588442	5	No	Yes	
CEBPA	mutation	AML	Diagnosis	B, BM	DNA	PCR or Sanger	2	588431-588442	5	No	Yes	
DNMT3A	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588442	5	No	Yes	
RUNX1	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588442	5	No	Yes	

Gene	Aberration type	Pathology	Phase	Sample types	DNA or RNA or nuclei	Techniques	Time impl.	Nomenclature billing codes	Max	Reimb. in practice	DNA-NGS possible?	Why not NGS?
RUNX1	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588442	5	No	Yes	
IDH1	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588443	5	No	Yes	
U2AF1	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588443	5	No	Yes	
ASXL1	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588443	5	No	Yes	
IDH2	multiple mutations	AML	Diagnosis	B, BM	DNA	Sanger or NGS	2	588431-588444	5	No	Yes	
RUNX1-RUNX1T1 / t(8;21)(q22;q22)	translocation	AML	Follow-up	B, BM	RNA	RT-PCR	0	588571-588582	1	Yes	No	Individual specific follow-up marker
CBFB-MYH11 / inv(16)(p13;q22)	translocation	AML	Follow-up	B, BM	RNA	RT-PCR	0	588571-588583	1	Yes	No	Individual specific
PML-RARA / t(15;17)(q22;q12)	translocation	AML	Follow-up	B, BM	RNA	RT-PCR	0	588571-588583	1	Yes	No	Individual specific follow-up marker
BCR-ABL1 / t(9;22)(q34;q11)	translocation	AML	Follow-up	B, BM	RNA	RT-PCR	0	588571-588584	1	Yes	No	Individual specific follow-up marker
DEK-NUP214 / t(6;9)(p23;q34)	translocation	AML	Follow-up	B, BM	RNA	RT-PCR	0	588571-588584	1	Yes	No	Individual specific follow-up marker
IGH	B-cel clonality	B-ALL	Diagnosis	B, BM, FT,	DNA	PCR	0	588490-588501	2	Yes	No	Distinct indication
IGH	B-cel clonality	B-ALL	Follow-up	B, BM	DNA	ASO-PCR	0	588571-588582	1	Yes	No	Distinct indication
MYC / 8q24	translocation	BL	Diagnosis	FFPE, FT	nuclei	FISH	0	588453-588464	3	Yes	No	Pathology-specific aberration
IGH	B-cel clonality	B-NHL	Diagnosis	B, BM, FT,	DNA	PCR	0	588475-588486	2	Yes	No	Distinct indication
HER2	amplification	Breast carcinoma	Diagnosis	FFPE	nuclei	ISH	0	588556-588560	1	Yes	No	ISH required for reimbursement
13q14.3	deletion	CLL/SLL	Diagnosis	B, BM, FT, FFPE	nuclei	FISH (+/- cell selection)	0	588453-588464	3	No	No	Other preferred technique
chr 12	trisomy	CLL/SLL	Diagnosis	B, BM, FT, FFPE	nuclei	FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique
11q22.3 (ATM)	deletion	CLL/SLL	Diagnosis	B, BM, FT, FFPE	nuclei	FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique
17p13.1 (TP53)	deletion	CLL/SLL	Diagnosis	B, BM, FT, FFPE	nuclei	FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique
NOTCH1	mutation	CLL/SLL	Diagnosis	B, BM, FT,	DNA	Sanger	2	588453-588464	3	No	Yes	
VH	multiple mutation	CLL/SLL	Diagnosis	B, BM, FT,	DNA	Sanger	0	no reimbursement		No	No	Distinct indication

Gene	Aberration type	Pathology	Phase	Sample types	DNA or RNA or nuclei	Techniques	Time impl.	Nomenclature billing codes	Max	Reimb. in practice	DNA-NGS possible?	Why not NGS?
BCR-ABL1 / t(9;22)(q34;q11)	translocation	CML	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	0	588512-588523	1	Yes	No	Pathology-specific aberration
BCR-ABL1 / t(9;22)(q34;q11)	translocation	CML	Follow-up	B, BM	RNA	RT-PCR	0	588593-588604	1	Yes	No	Individual specific follow-up marker
BRAF V600E	mutation	Colon carcinoma	Diagnosis	FFPE	DNA	PCR, Sanger, Pyrosequencing or NGS	2	588534-588545	2	No	Yes	
NRAS	multiple mutations	Colon carcinoma	Diagnosis	FFPE	DNA	PCR, Sanger, Pyrosequencing or NGS	2	588534-588545	2	Yes	Yes	
KRAS	multiple mutations	Colon carcinoma	Diagnosis	FFPE	DNA	PCR, Sanger, Pyrosequencing or NGS	1	589713-589724	1	Yes	Yes	
MYD88 L265P	mutation	DLBCL	Diagnosis	B, BM, FT, FFPE	DNA	PCR or Sanger	2	588453-588464	3	No	No	Pathology-specific aberration
MYC / 8q24	translocation	DLBCL	Diagnosis	FFPE, FT	nuclei	FISH	0	588453-588464	3	Yes	No	Other preferred technique
BCL6 / 3q27	translocation	DLBCL	Diagnosis	FFPE, FT	nuclei	FISH	0	588453-588464	3	Yes	No	Other preferred technique
IGH-BCL2 / t(14;18)(q32;q21)	translocation	DLBCL	Diagnosis	B, BM, FT, FFPE	DNA or nuclei	PCR or FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique
IGH-BCL2 / t(14;18)(q32;q21)	translocation	DLBCL	Follow-up	B, BM	DNA or nuclei	PCR or FISH (+/- cell selection)	0	588571-588582	1	Yes	No	Pathology-specific aberration
IGH-BCL2 / t(14;18)(q32;q21)	translocation	FL	Diagnosis	B, BM, FT, FFPE	DNA or nuclei	PCR or FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Pathology-specific aberration
IGH-BCL2 / t(14;18)(q32;q21)	translocation	FL	Follow-up	B, BM	DNA or nuclei	PCR or FISH (+/- cell selection)	0	588571-588582	1	Yes	No	Pathology-specific aberration
HER2	amplification	Gastric carcinoma	Diagnosis	FFPE	nuclei	ISH	2	588534-588545	2	Yes	No	Other preferred technique
KIT	multiple mutations	GIST	Diagnosis	FFPE	DNA	Sanger or NGS	2	588534-588545	2	Yes	Yes	
PDGFRA	multiple mutations	GIST	Diagnosis	FFPE	DNA	Sanger or NGS	2	588534-588545	2	Yes	Yes	

Gene	Aberration type	Pathology	Phase	Sample types	DNA or RNA or nuclei	Techniques	Time impl.	Nomenclature billing codes	Max	Reimb. in practice	DNA-NGS possible?	Why not NGS?
1p36	deletion	Glioma	Diagnosis	FFPE	nuclei	FISH	2	588534-588545	2	Yes	No	Other preferred technique
19q13	deletion	Glioma	Diagnosis	FFPE	nuclei	FISH	2	588534-588545	2	Yes	No	Other preferred technique
IDH1	mutation	Glioma	Diagnosis	FFPE	DNA	PCR, Sanger or NGS	2	588534-588545	2	No	Yes	
IDH2	mutation	Glioma	Diagnosis	FFPE	DNA	PCR, Sanger or NGS	2	588534-588545	2	No	Yes	
BRAF V600E	mutation	HCL	Diagnosis	B, BM, FT, FFPE	DNA	PCR or Sanger	2	588453-588464	3	Yes	No	Pathology-specific aberration
MYD88 L265P	mutation	LPL	Diagnosis	B, BM, FT, FFPE	DNA	PCR or Sanger	2	588453-588464	3	Yes	No	Pathology-specific aberration
ALK / 2p23	translocation	Lung carcinoma	Diagnosis	FFPE	DNA	FISH or RT-PCR	2	588534-588545	2	No	No	Other preferred technique
ROS1	translocation	Lung carcinoma	Diagnosis	FFPE	DNA	FISH	2	588534-588545	2	No	No	Other preferred technique
EGFR	multiple mutations	Lung carcinoma	Diagnosis	FFPE	DNA	PCR, Sanger, Pyrosequencing or NGS	2	588534-588545	2	Yes	Yes	
KIT	multiple mutations	Malignant melanoma	Diagnosis	FFPE	DNA	Sanger or NGS	2	588534-588545	2	No	Yes	
PDGFRA	multiple mutations	Malignant melanoma	Diagnosis	FFPE	DNA	Sanger or NGS	2	588534-588545	2	No	Yes	
BRAF V600E	mutation	Malignant melanoma	Diagnosis	FFPE	DNA	PCR, Sanger, Pyrosequencing or NGS	2	588534-588545	2	Yes	Yes	
NRAS	multiple mutations	Malignant Melanoma	Diagnosis	FFPE	DNA	PCR, Sanger, Pyrosequencing or NGS	2	588534-588545	2	Yes	Yes	
MALT1 / 18q21	translocation	MALT-L	Diagnosis	FFPE	nuclei	FISH	0	588453-588464	3	Yes	No	Pathology-specific aberration
KIT D816V	mutation	Mastocytosis	Diagnosis	B, BM	DNA	PCR, Sanger or NGS	2	no reimbursement		No	Yes	
IGH-CCND1 / t(11;14)(q13;q32)	translocation	MCL	Diagnosis	B, BM, FT, FFPE	DNA or nuclei	PCR or FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Pathology-specific aberration
IGH-CCND1 / t(11;14)(q13;q32)	translocation	MCL	Follow-up	B, BM	DNA or nuclei	PCR or FISH (+/- cell selection)	0	588571-588582	1	Yes	No	Individual specific follow-up marker

Gene	Aberration type	Pathology	Phase	Sample types	DNA or RNA or nuclei	Techniques	Time impl.	Nomenclature billing codes	Max	Reimb. in practice	DNA-NGS possible?	Why not NGS?
TP53	multiple mutations	MDS	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement		No	Yes	
IDH1	multiple mutations	MDS	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement	5	No	Yes	
IDH2	multiple mutations	MDS	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement	5	No	Yes	
SRSF2	multiple mutations	MDS/CMML	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement		No	Yes	
TET2	multiple mutations	MDS/CMML	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement		No	Yes	
SF3B1	multiple mutations	MDS-RARS/ RCMD-RS	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement		No	Yes	
FIP1L1-PDGFRA	translocation	MPN	Diagnosis	B, BM	RNA or nuclei	RT-PCR or FISH	2	no reimbursement		No	No	RNA analysis
CALR	mutation	MPN	Diagnosis	B, BM	DNA	PCR, Sanger or NGS	2	no reimbursement		No	Yes	
MPL	mutation	MPN	Diagnosis	B, BM	DNA	PCR, Sanger or NGS	2	no reimbursement		No	Yes	
CSFR3	multiple mutations	MPN	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement		No	Yes	
JAK2 exon 12	mutation	MPN	Diagnosis	B, BM	DNA	Sanger or NGS	2	no reimbursement		No	yes	
JAK2 V617F	mutation	MPN	Diagnosis	B, BM	DNA or RNA	PCR or RT-PCR	1	589691-589702	1	Yes	Yes/No	Distinct indication
IGH	B-cel clonality	NHL	Follow-up	B, BM	DNA	PCR	0	588571-588582	1	Yes	No	Distinct indication
IGH-CCND1 / t(11;14)(q13;q32)	translocation	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	0	588453-588464	3	No	No	Other preferred technique
IGH-MAFB / t(14;20)(q32;q12)	translocation	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	2	588453-588464	3	No	No	Other preferred technique
chr 1q	duplication/ amplification	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	2	588453-588464	3	No	No	Other preferred technique
13q14 (RB1)	deletion	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	0	588453-588464	3	No	No	Other preferred technique
IGH-FGFR3 / t(4;14)(p16;q32)	translocation	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique
IGH-MAF / t(14;16)(q32;q23)	translocation	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique
17p13.1 (TP53)	deletion	PCM	Diagnosis	BM	nuclei	FISH (+/- cell selection)	0	588453-588464	3	Yes	No	Other preferred technique

Gene	Aberration type	Pathology	Phase	Sample types	DNA or RNA or nuclei	Techniques	Time impl.	Nomenclature billing codes	Max	Reimb. in practice	DNA-NGS possible?	Why not NGS?
SS18 / 18q11.2	translocation	Sarcoma	Diagnosis	FFPE	nuclei	FISH	0	588534-588545	2	Yes	No	Other preferred technique
DDIT3 / 12q13	translocation	Sarcoma	Diagnosis	FFPE	nuclei	FISH	0	588534-588545	2	Yes	No	Other preferred technique
FUS / 16p11	translocation	Sarcoma	Diagnosis	FFPE	nuclei	FISH	0	588534-588545	2	Yes	No	Other preferred technique
MDM2	amplification	Sarcoma	Diagnosis	FFPE	nuclei	FISH	0	588534-588545	2	Yes	No	Other preferred technique
TCR	T-cel clonality	T-ALL	Diagnosis	B, BM, FT,	DNA	PCR	0	588490-588501	2	Yes	No	Distinct indication
TCR	T-cel clonality	T-ALL	Follow-up	B, BM	DNA	PCR	0	588571-588582	1	Yes	No	Distinct indication
TCR	T-cel clonality	T-NHL	Diagnosis	B, BM, FT,	DNA	PCR	0	588475-588486	2	Yes	No	Distinct indication
TCR	T-cel clonality	T-NHL	Follow-up	B, BM	DNA	PCR	0	588571-588582	1	Yes	No	Distinct indication

APPENDIX 5. SELECTED NOMENCLATURE CODES

Couple	Out-patient	In-patient	Dutch description	French description	In use since	Suppressed since	Last modification
32_a	588070	588081	Immunohistologische onderzoeken (maximum 4 per afname) voor het aantonen van antigenen in de coupes, na incubatie met antisera, per gebruikt antiserum	Examens immunohistologiques (maximum 4 par prélèvement) pour révéler des antigènes sur des coupes, après incubation d'anticorps, par anti-sérum	1/04/1985		1/07/1999
32_b	588976	588980	Honorarium voor de immunohistologische onderzoeken voor het aantonen van farmaco-diagnostiche antigenen in de coupes na incubatie met antisera, per gebruikt antiserum, in het kader van het voorschrijven van tumour-specifieke medicatie bij oncologische patiënten	Honoraires pour les examens immuno-histologiques pour la mise en évidence d'antigènes pharmaco-diagnostiques au niveau des coupes, après incubation avec antisérums, par antisérum utilisé, dans le cadre de la prescription d'une médication spécifique à la tumeur pour des patients oncologiques	1/07/2009		
33_l	588615	588626	Karyogram (andere gevallen dan die bedoeld onder verstrekking nr. 588652 - 588663)	Caryogramme (autres cas que ceux prévus à la prestation n° 588652 - 588663)	1/04/1985	1/01/2013	
33_m	588696	588700	Opzoeken van genetische anomalieën volgens de methoden van hybridisatie van DNA-fragmenten	Recherche d'anomalies génétiques par les méthodes d'hybridation de fragments d'A.D.N.	1/04/1986	1/01/2013	
33b_a	588431	588442	Opsporen van verworven chromosoom of genafwijkingen (met uitsluiting van immuunglobulineg of een T-celreceptorgenherschikking), door middel van een moleculair biologische methode : in de diagnostische investigatiefase van een acute leukemie, inclusief Burkitt's lymfoom of T- of B- lymfoblastisch lymfoom of refractaire anemie met blastenoverproductie (RAEB) (Diagnoseregel 1, 5)	Dépistage d'anomalies acquises chromosomiques ou géniques (à l'exception du réarrangement des gènes des immunoglobulines ou des gènes du récepteur des cellules T), au moyen d'une méthode de biologie moléculaire dans la phase d'investigation diagnostique d'une leucémie aiguë, y compris le lymphome de Burkitt ou le lymphome T- ou B-lymphoblastique ou l'anémie réfractaire avec excès de blastes (AREB) (Règle diagnostique 1, 5)	1/08/2007		1/08/2010
33b_b	588453	588464	Opsporen van verworven chromosoom of genafwijkingen (met uitsluiting van immuunglobuline- of een T-celreceptorgenherschikking), door middel van	Dépistage d'anomalies acquises chromosomiques ou géniques (à l'exception du réarrangement des gènes des immunoglobulines ou des gènes du récepteur	1/08/2007		1/08/2010

			een moleculair biologische methode : in de diagnostische investigatiefase van een chronische lymfoïde aandoening (non-Hodgkin lymfoom, chronische lymfatische leukemie, multiple myeloom), exclusief een acute leukemie, Burkitt's lymfoom of T- of B-lymfoblastisch lymfoom en refractaire anemie mey blastnoverproductie (RAEB) (Diagnoseregel 1, 6)	cellule T), au moyen d'une méthode de biologie moléculaire : dans la phase d'investigation diagnostique d'une affection lymphoïde chronique (lymphome non-Hodgkinien, leucémie lymphoïde chronique, myélome multiple), à l'exclusion d'une leucémie aiguë, du lymphome de Burkitt, ou des lymphomes lymphoblastiques B ou d'une anémie réfractaire avec exès de blaste (AREB) (Règle diagnostique 1, 6)		
33b_c	588475	588486	Opsporen van een immuunglobulinegen- of een T-celreceptorgenherschikking met een moleculair biologische methode : in de diagnostische investigatiefase van een chronische lymfatische leukemie of van een non-Hodgkin's lymfoom (exclusief een acute leukemie, Burkitt's lymfoom of T- of B-lymfoblastisch lymfoom) (Diagnoseregel 1, 7)	Dépistage du réarrangement des gènes des immunoglobulines ou des gènes du récepteur -T au moyen d'une méthode de biologie moléculaire : dans la phase d'investigation diagnostique d'une leucémie lymphoïde chronique ou d'un lymphome non-Hodgkinien (à l'exclusion d'une leucémie aiguë, d'un lymphome de Burkitt ou de lymphomes lymphoblastiques T- ou B) (Règle diagnostique 1, 7)	1/08/2007	1/08/2010
33b_d	588490	588501	Opsporen van een immuunglobulinegen- of een T-celreceptorgenherschikking met een moleculair biologische methode : in de diagnostische investigatiefase van een acute lymfoblasten leukemie, Burkitt's lymfoom of T- of B- lymfoblastisch lymfoom of refractaire anemie met blastenoverproductie (AREB) (Diagnoseregel 1, 7)	Dépistage du réarrangement des gènes des immunoglobulines ou des gènes du récepteur -T au moyen d'une méthode de biologie moléculaire : dans la phase d'investigation diagnostique d'une leucémie lymphoblastique aiguë, d'un lymphome de Burkitt ou de lymphomes lymphoblastiques -T ou -B ou de l'anémie réfractaire avec excès de blastes (AREB) (Règle diagnostique 1, 7)	1/08/2007	1/08/2010
33b_e	588512	588523	Opsporen van verworven chromosoom of genafwijkingen (met uitsluiting van immuunglobuline- of een T-celreceptorgenherschikking), door middel van een moleculair biologische methode : in de diagnostische investigatiefase van een chronische myeloïde leukemie (Diagnoseregel 1, 13)	Dépistage d'anomalies acquises chromosomiques ou géniques (à l'exception des réarrangements des gènes des immunoglobulines et du récepteur -T) au moyen d'une méthode de biologie moléculaire : dans la phase d'investigation diagnostique d'une leucémie myéloïde chronique (Règle diagnostique 1, 13)	1/08/2007	1/08/2010

33b_f	588534	588545	Opsporen van een verworven chromosoom of genafwijking door middel van een moleculair biologische methode, in de diagnostiche investigatiefase van een niet-lymfoïde en niet-myeloïde vaste tumour (Diagnoseregel 1, 8)	Dépistage d'anomalies chromosomiques ou géniques acquises au moyen d'une méthode de biologie moléculaire, dans la phase d'investigation diagnostique d'une tumeur solide non-lymphoïde et non-myéloïde (Règle diagnostique 1, 8)	1/08/2007	1/08/2010
33b_g	588556	588560	Opsporen van HER2 genamplificatie door middel van een in situ "hybridization" techniek voor therapiekeuze bij mammacarcinoom in de diagnostische investigatiefase (Diagnoseregel 1, 13)	Dépistage d'une amplification du gène HER2 au moyen d'une technique par "hybridization" in situ dans le cadre du choix thérapeutique pour le carcinome mammaire (Règle diagnostique 1, 13)	1/08/2007	1/07/2011
33b_h	589691	589702	Opsporen van de JAK2 mutatie V617F in de diagnostische investigatiefase van een myeloproliferatief syndroom (MPS) (Diagnoseregel 1,13)	Dépistage de la mutation V617F de JAK2 dans la phase d'investigation diagnostique d'un syndrome myéloprolifératif (SMP) (Règle diagnostique 1, 13)	1/08/2010	
33b_i	589713	589724	Opsporen van een verworven afwijking van het K-RASgen door middel van een moleculair biologische methode in het kader van het voorschrijven van een behandeling door tumour-specifieke monoklonale antilichamen bij patiënten met een gemetastaseerd colorectaal carcinoom (Diagnoseregel 14, cumulregel 1)	Dépistage d'une anomalie acquise du gène K-RAS par méthode de biologie moléculaire, dans le cadre de la prescription d'un traitement par anticorps monoclonaux spécifiques de la tumeur chez des patients présentant un carcinome colorectal métastasé (Règle diagnostique 14, règle de cumul 1)	1/05/2011	
33b_j	588571	588582	Opsporen van verworven chromosoom of genafwijkingen door middel van een moleculair biologische methode als opvolging van een lymfoïde of myeloïde aandoening, met uitzondering van een chronische myeloïde leukemie, waarbij de betreffende afwijkingen in de diagnostische investigatiefase zijn vastgesteld, en waarbij een therapie met curatief doeleinde is ingesteld (Maximum 1) (Diagnoseregel 9)	Dépistage d'anomalies acquises chromosomiques ou géniques au moyen d'une méthode de biologie moléculaire pour le suivi d'une affection lymphoïde ou myéloïde, à l'exception d'une leucémie myéloïde chronique, pour laquelle les anomalies concernées ont été établies dans la phase d'investigation diagnostique et pour laquelle un traitement à but curatif est instauré (Maximum 1) (Règle diagnostique 9)	1/08/2007	1/08/2010
33b_k	588593	588604	Opsporen van een verworven genherschikking door middel van een kwantitatieve moleculaire biologische methode als opvolging van een chronische	Dépistage d'un réarrangement de gène acquis, au moyen d'une méthode quantitative de biologie moléculaire pour le suivi d'une leucémie myéloïde chronique dans laquelle	1/08/2007	1/08/2010

			myeloïde leukemie waarbij een bcr/alb genherschikking in de diagnostische investigatiefase is vastgesteld, en waarbij een therapie met curatief doeleinde is ingesteld (Maximum 1) (Diagnoseregel 9)	un réarrangement du gène bcr/abl a été établi dans la phase d'investigation diagnostique, et pour laquelle un traitement à but curatif est instauré (Maximum 1) (Règle diagnostique 9)		
33b_l	588770	588781	Opsporen van een verworven genafwijking in beenmerg door middel van een moleculair biologische methode, als opvolging van een gemetastaseerde niet-lymfoïde en niet-myeloïde vaste tumour, waarin de betreffende genherschikking in de diagnostische investigatiefase is vastgesteld, en waarbij een therapie met curatief doeleinde is ingesteld (Maximum 1) (Diagnoseregel 10)	Dépistage d'une anomalie génique acquise dans la moelle osseuse, au moyen d'une méthode de biologie moléculaire, pour le suivi d'une tumeur solide métastasée non-lymphoïde et non-myéloïde dont le réarrangement de gène concerné a été établi dans la phase d'investigation diagnostique, et pour lequel un traitement à but curatif est instauré (Maximum 1) (Règle diagnostique 10)	1/08/2007	1/08/2010

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