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Pre-Examination Factors Affecting Molecular Diagnostic Test Results and Interpretation: a Case-Based Approach.

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1. Introduction

Clinical molecular diagnostic laboratories utilize international, national, regional and professional guidance documents to insure quality and safety. The variety of guidelines and the purpose of specific aspects of such guidelines can be difficult to navigate. In order to educate and support molecular diagnostic laboratories, the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) Committee on Molecular Diagnostics (C-MD) performed an informal survey of its international members to identify the most commonly utilized guidance document for clinical laboratories [1]. The most cited guidance document by the IFCC C-MD expert panel representing molecular diagnostic laboratories performing testing for infectious disease, oncology, inherited disease and pharmacogenomics was the International Organization for Standardization (ISO) 15189 standard. ISO 15189 describes both management practices (for example, quality management systems) and technical practices for medical laboratories (for example, pre-examination, examination and post-examination processes) [2]. Several studies demonstrate that case-based teaching improves the educational effectiveness of presentations [3, 4]. For this reason, clinical vignettes can be used to educate laboratories on the utility of specific aspects of the recommendations for laboratories performing molecular diagnostics. Selected case-based examples in the pre-examination phase for molecular laboratory testing include information provided to patients and users, requisitions, primary sample collection/handling, informed consent and maintaining sample integrity upon receipt into the laboratory and prior to testing. In this opinion paper, cases (specifically, inherited diseases, oncology, pharmacogenomics, and infectious diseases) illustrate the value of applying recommendations for the pre-examination phase to the more specialized area of Molecular Diagnostics.

2. Material and Methods

Through a collaborative process with an international molecular diagnostic expert panel, a survey identified and ranked the guidelines used by molecular laboratories located in Australia, Canada, France, Germany, Israel, Japan, Paraguay, Slovakia and the United States of America. Guidelines cited ranged from general laboratory, international, national, discipline specific, and province/state specific documents [5-7]. Specifically, the International Organization for Standardization (ISO), Clinical and Laboratory Standards Institute (CLSI), Canadian Standards Association (CSA), Comité français d'accréditation (COFRAC), New York Department of Health (NYDOH), Australian Government Department of Health Therapeutic Goods Administration, Slovak National Accreditation Service (SNAS) and the American College of Medical Genetics (ACMG) were mentioned as resources for laboratories performing molecular diagnostics [5, 7-11]. Additional guidance documents and reports not identified during the survey can be found on the IFCC C-MD website [12]. The most frequently cited guidance document was ISO 15189. ISO 15189 includes management and technical practices for all types of medical laboratories. The pre-examination portion was selected by the panel for discussion. Case-based examples are designed to illustrate the value of utilizing the guidelines for improving patient care. Some of the examples discussed below may not currently be part of best practice guidelines nor formally approved by Health-Technology-Assessment (HTA) bodies, but the case-based examples illustrate the importance of pre-examination (also known as pre-analytical) variables and components on the quality of molecular tests results.

3. Information for Patients and Users

Educating patients and healthcare providers on the requirements, purposes and limits of laboratory testing promotes appropriate test utilization. Appropriate test utilization includes avoiding unnecessary testing and, therefore, produces cost savings to the healthcare system. Additionally, adverse effects of testing such as repeated testing due to inappropriate test selection and discomfort to the patient can be avoided. Listed below are examples illustrating outcomes when the guidelines are either followed or not followed. As specified by ISO 15189, the laboratory should provide information on acceptable specimen types and volumes, turn-around- times (TAT), specimen transportation requirements, rejection criteria and factors impacting test performance and interpretation, and access to laboratory personnel capable of providing clinical advice on test ordering and interpretation. Educational resources may include laboratory's instructions on patient preparation and sampling specifications, comments on laboratory results, access to laboratory personnel, and/or web based resources for the provider.

3.1. *Molecular infectious disease: Sample type and transportation.*

3.1.1. Background: Group B Streptococcus (GBS) screening should be performed less than or equal to 5 weeks prior to childbirth. The Centers for Disease Control (CDC) guideline requires that this specimen has an enrichment step to avoid potential false negative results. For these reasons, GBS samples must be collected using media that permits culture prior to molecular testing. The specimen needs to be viable and the specific collection necessitates both rectal and vaginal collections. The collected specimen should be stored at 4°C to maintain viability [13].

3.1.1.1. Case-based example: Specimens collected for GBS testing were placed in a box on the exterior of a clinic's facility facing west. The west facing box became very hot during the day. The laboratory courier noticed that the samples were hot to the touch and documented their observation. The specimen did not grow in the enrichment media presumably because the pre-examination storage caused decreased viability in the sample. Because no visible growth was apparent, molecular testing could not be performed and it was reported as indeterminate. A comment was included in the report explaining possible causes for inadequate growth.

3.1.1.2. Case-based example: Testing was requested on a vaginal sample that was collected using a liquid based preservative containing an alcohol fixative. The specimen was rejected for examination because the fixative prevented the culture based enrichment step prior to molecular testing and the specimen collection did not include a rectal collection. The client services employee contacted the healthcare provider that the sample had been rejected.

3.1.1.3. Case-based example: A clinician contacted the laboratory requesting information on vaginal collection without rectal collection. The laboratory client services department informed the healthcare provider of the risk for potential false negative results if sample collection was from only one site.

3.2 *Molecular Oncology: Sample type, factors influencing test performance and healthcare provider education*

3.2.1 Background: Screening for clinically important Epidermal Growth Factor Receptor (*EGFR*) mutations in solid tumors (specifically, non-small cell lung cancer) should be performed within 2 weeks of sample reception. The American Thoracic Society and the European Respiratory Society recommend dissection of formalin fixed paraffin embedded (FFPE) tissues to enrich the tumor content in the tested specimen and hence to avoid false negative results. Because test performance depends on the tumor content of the FFPE specimen, patient samples should usually contain more than 10 % tumor tissue and be stored at room temperature. The most representative FFPE block selected by pathologist should be used for molecular testing [14, 15]. These requirements were described in the test directory provided by the laboratory to the healthcare providers.

3.2.1.1. Case-based example: The FFPE specimen was evaluated by a pathologist. Due to low content of tumor tissue (specifically, less than 10%) and high content of necrotic, stromal and benign components, the sample was reported as being unrepresentative. Subsequently, macrodissection of the specimen performed in the laboratory achieved enrichment of tumor of up to 50%. Molecular testing of *EGFR* mutational status was performed without compromising the result outcome. The healthcare provider was contacted by the laboratory and educated on the testing requirements for tissue biopsies for future testing.

3.2.1.2. Case-based example: An FFPE specimen was prepared after the first chemotherapy treatment by endobronchial ultrasound-guided- fine needle aspiration and provided to the laboratory. The test was rejected for further examination since the low content of malignant cells did not satisfy the test requirements. The healthcare provider was contacted and informed that the sample quantity was insufficient for testing.

3.2.1.3. Case-based example: A tissue sample was collected and inadvertently placed in a saline solution for an extended timeframe in the operating room. Subsequently, the operating room personnel submitted the sample in formalin where it was delivered to the laboratory and processed. Following histological examination, the sample satisfied the pathologist's criteria; yet, the analysis failed. Examination of the isolated DNA demonstrated extensively fragmented, low quality DNA, presumably caused by the delay in placing the specimen in formalin. The laboratory retested the sample using methodology adjusted for samples containing DNA fragments of approximately 120 base pairs (bp). The retested sample satisfied all control reactions and the test result on *EGFR* genotyping was reported. The report had an amended comment with a comment describing the use of a different method.

3.2.2. Background: *BRAF* also known as Serine/threonine-protein kinase B-raf regulates the MAP kinase/ERKs signaling pathway. The *BRAF* p.V600E mutation (also referred to as the *BRAF* c.1799T > A mutation) is associated with a variety of cancers including melanoma. Detection of this variant may guide the decision to prescribe selective agents such as Vemurafenib [16, 17]. The collection requirements for testing melanoma biopsies were available in the test directory provided by the laboratory to the healthcare providers.

3.2.2.1. Case-based example: Testing for the *BRAF* c.1799T > A was conducted using DNA extracted from a FFPE specimen derived from a melanoma patient. The quantitative polymerase chain reaction (qPCR)

assay did not detect the mutation or the internal PCR control; thus, the sample was reported as indeterminate. The clinician was informed of the test limitation for samples containing high amounts of melanin (specifically, more than 0.15 ug/mL melanin in this particular case). Samples containing high amounts of melanin yield indeterminate results by both immunohistochemistry (IHC) and qPCR [18, 19]. A request for sample containing less pigmented skin area was sent to the attending physician.

3.2.3. Background: Molecular detection of *BCR-ABL* gene rearrangement by reverse transcriptase (RT) qPCR has both diagnostic and prognostic value for patients with Chronic Myelogenous Leukemia (CML) [20]. Log decreases of mRNA levels of the *BCR-ABL* are predictive of response to treatment. In contrast, monitoring of increases in mRNA may be indicative of relapse or the development of resistance.

3.2.3.1. Case-based example: A CML patient was scheduled for qPCR monitoring of the *BCR-ABL* gene rearrangement. The patient received a blood transfusion and the healthcare provider cancelled the test. A blood transfusion may affect the ability of the laboratory to monitor therapeutic failure and was correctly cancelled by the healthcare provider.

3.2.3.2. Case-based example: Bone marrow or peripheral blood may be used as the specimen when monitoring *BCR-ABL* gene rearrangement by qPCR. The user needs to be informed if the sample type collected for monitoring therapeutic response differs from that used for diagnosis. For example, the diagnostic sample for CML is often a bone marrow aspirate. The ratio of *BCR-ABL* gene rearrangement to the reference gene (also known as, the housekeeping gene) may differ between a peripheral blood and a bone marrow aspirate because the cellularity may be higher due the presence of stromal cells in the bone marrow sample. Informing the user or healthcare provider, that both a peripheral and bone marrow sample may be collected at the time of diagnoses permits follow up disease monitoring using a less invasive sample such as peripheral blood [21].

3.3. *Inherited disease: Patient history and biogeographical ancestral history*

3.3.1 Background: Health care providers need to be aware of the limitations of laboratory testing for certain patients. For example, in the context of prenatal testing, the patient history such as gestational age and maternal health may factor into the diagnostic value of certain molecular tests. Rhesus D (RhD) factor is a protein on red blood cells. Patients who have this factor are referred to as being RhD positive versus RhD negative. When pregnant patients are RhD negative and their baby is RhD positive, adverse pregnancy events can occur. The woman may develop antibodies that attack the fetus's blood cells resulting in anemia or fetal death [22, 23]. The limitations of testing may be communicated to the healthcare provider and/or included as part of the requisition form (later discussed in section 4).

3.3.1.1. Case-based Example: Noninvasive prenatal testing (NIPT) for detecting rhesus D (RhD) incompatibility, chromosomal aneuploidies and monogenic diseases was requested on a cell-free plasma sample from a pregnant woman. The sample was rejected and the healthcare provider contacted because no information regarding the gestational age and the last menstrual period of the pregnant woman was provided. The accuracy of these tests is dependent on the amount of fetal DNA in the maternal plasma sample being tested. Although fetal DNA can be detected in maternal plasma as early as 4 weeks of gestation, up to 10% of maternal plasma samples can have an undetectable level of

fetal DNA [24]. The minimum fractional concentration of fetal DNA required has not been established at the present time due to variability between methods. With this said, it is generally accepted that at least 4% fetal DNA, based on the comparison of the number of genome-equivalents, needs to be present in maternal plasma samples to allow reliable detection of chromosomal aneuploidies.

In addition to gestational age, maternal weight has also been shown to correlate negatively with the fetal DNA fraction [25]. The estimated proportion of samples with <4% fetal DNA increased with maternal weight from 0.7% at 60kg to 7.1% at 100kg. Thus, information on gestational age, multiple pregnancy and maternal weight is an essential consideration for NIPT.

3.3.2. Background: Educating healthcare providers on the utility of genetic panels for specific patients insures that appropriate testing is performed. Provision of family history is helpful in assisting the laboratory and clinicians in determining which alleles/methods need to be tested. Providing this information to the user avoids delays in service and reduces unnecessary laboratory costs. The patient's population of origin is important information that needs to be provided prior to genetic testing. Frequently, variant panels (also referred to as mutation panels) are designed based on observed variants found within the population to be tested, and may even be reduced to a few founder mutations [26-29]. Thus, both the healthcare provider and the laboratory need the patient's biogeographical ancestry information so that accurate mutation rates are calculated, interpreted, and reported.

3.3.2.1. Case-based example: A patient from Ashkenazi decent relocated to Quebec City, Canada and requested genetic screening for cystic fibrosis (CF). The healthcare provider following consultation with the laboratory chose a population specific panel for this patient in order to increase the diagnostic value of the laboratory results versus the French-Canadian CF panel. For instance, a panel that detects over 95% of mutations in French-Canadians that have a strong founder effect may perform poorly in patients from other origins [30, 31].

3.4. Pharmacogenomic (PGx): Turn around time (TAT) expectations.

3.4.1 Background: Appropriate TATs are essential for determining whether the test result can be used in a clinical setting. If a pharmacogenomic test is requested, for example to explain aberrant findings in patient outcome or drug concentration measurements, a TAT within one week is advisable. The one week TAT ensures healthcare providers adequate time to improve management of patient care. Since some pharmacogenomic tests are optimal when used to predict the response (prospective use), the TAT should be within one week, and for some applications even faster. This means adjusting the test TAT to comply with clinical logistics, or vice versa. Collaboration with healthcare providers is advised when determining the specific patient management needs for a pharmacogenomic test and for setting expectations for laboratory service.

3.4.1.1. Case-based example: Prior to scheduling a patient for chemotherapy with Capecitabine, the patient's blood sample is collected and sent the laboratory for DPYP genotyping. Capecitabine (an active compound containing 5-Fluorouracil) is an anti-metabolic chemotherapeutic agent used to treat patients with breast or colorectal cancer [32]. Patients with dihydropyrimidine dehydrogenase (DPYD) deficiency will experience a higher risk of toxicity to capecitabine [33]. For this reason, DPYP genotyping

may be requested prior to starting capecitabine therapy, since such testing may predict 50% of PDYP deficient patients. Hospital-based testing generally requires results to be available to the healthcare provider within 3-4 working days. Failure to deliver this TAT will have consequences for the patient's health since their chemotherapy will either be delayed, or started without genotype information.

3.4.1.2. Case-based example: A patient from Shanghai, China presents with a symptoms consistent with stroke. The clinician is considering a variety of medications but is concerned that patient's Chinese ancestry may influence the efficacy of the medication. The healthcare provider contacts the molecular laboratory to determine the TAT of pharmacogenomic testing for the *CYP2C19* gene. With a TAT of less than 24 hours, the healthcare provider orders the test and proceeds with prescribing Clopidogrel. Clopidogrel (also known as Plavix) is a platelet aggregation inhibitor used to reduce the risk for stroke or heart attacks in patients with a history of either of these conditions. About 2% of white patients, 4% of black patients, and 14% of Chinese patients do not convert the prodrug to the active form [34]. The *CYP2C19* gene is responsible for conversion of the prodrug by cytochrome P450 2C19 and has several variants associated with different levels of activity. To ensure efficacious use of the drug, the *CYP2C19* test that is performed with a TAT of less than 24 hours provides actionable information for future dosage adjustments.

3.4.1.3. Case-based example: A patient presents with shortness of breath, sharp pains and other symptoms consistent with pulmonary embolism. Following emergency treatment with IV Heparin, the healthcare provider considers prescribing warfarin in this patient but is also concerned with adverse reactions to the drug. The healthcare provider proceeds with prescribing the warfarin but also orders molecular tests for *CYP2C9* and *VKORC1*. Warfarin (also known as Coumadin) is an anticoagulant that is used to reduce the risk for blood clots in some patients. Genetic variants in *CYP2C9* encoding cytochrome P450 2C9 influence the ability to metabolize the *S*-enantiomer to the active 7-hydroxywarfarin. A genetic variant in the vitamin K epoxide reductase complex (*VKORC1*) is associated with increased sensitivity to the drug and may affect the optimal dosing of warfarin per patient [35, 36]. The main adverse outcome associated with inappropriate dosing is increased risk of severe bleeding. In order to improve patient outcomes, the TAT for *CYP2C9/VKORC1* should be within 2-3 days. This TAT is based on the typical plateau phase of drug levels.

4. Request Form Information

The request form or requisition should include relevant information and is acquired by the laboratory to ensure appropriate testing and interpretation. The specimen type and, in some cases, the anatomic site of sample origin should be included. Healthcare providers should use appropriate nomenclature and include additional information to guide the laboratory in the processing and testing of the sample. Request forms with sufficient fields and space to either designate or add clinically relevant information are useful and may include ancestry, family history, travel and history of exposure to communicable diseases. Clinical information such as the rationale and purpose of the molecular diagnostic test provides important contextual information for appropriate test interpretation by laboratory personnel.

4.1. Molecular Infectious Disease: Anatomical site, clinical history, purpose of the test, travel history and test interpretation

4.1.1. Background: Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) are found in mucocutaneous sites including the oral cavity, and genital region. The laboratory validated their HSV-1 and HSV-2 molecular tests on mucocutaneous lesions in the oral and genital area [37].

4.1.1.1 Case-based example: The laboratory received a request form and a collection device for HSV-1 and HSV-2 PCR based testing. The requisition form stated that the sample collection was from a corneal ulcer. The laboratory rejected the test request because the laboratory's validation did not include an assessment of corneal lesion samples.

4.1.2. Background: *Chlamydia trachomatis*(CT) and *Neisseria gonorrhoeae*(NG) are sexually transmitted infectious agents. The diagnostic approach for clinical purposes differs from the diagnostic approach in cases of sexual abuse or other forensic applications.

4.1.2.1. Case-based example: The laboratory received a request form for CT/NG testing. The requisition stated that the patient had been raped. The laboratory notified the user that the sample could not be used as forensic evidence. The user and patient decided to continue with the laboratory analysis but also attended an emergency room for forensic evidence collection [38].

4.1.3 Background: Leishmaniasis is a disease caused by protozoans from the genus *Leishmania*. Approximately, 21 different species of *Leishmania* are associated with human disease [39]. The species are distributed in different geographical locations (specifically, Asia, the Middle East, Africa, Southern Europe, Central and South America). There are three main forms of the disease: cutaneous, visceral or kala-azar and mucocutaneous. The skin sores of cutaneous leishmaniasis usually develop within a few weeks or months of the sand fly bite. People with visceral leishmaniasis usually become sick within months of the bite, and occasionally years later. In the case of visceral leishmaniasis, presentation includes fever, weight loss, enlargement (swelling) of the spleen and liver, and abnormal blood tests such as low blood counts, including low red blood cell (anemia), white blood cell (leukopenia), and platelet (thrombocytopenia) counts.

4.1.3.1. Case-based example: A patient presents with one or more skin sores, as papules (bumps) or nodules (lumps) or skin ulcers covered by scab or crust. The healthcare provider performs a physical exam and determines that the patient traveled to the Amazon region of Brazil six weeks earlier [40]. The travel history was communicated to the laboratory on the requisition. Tissue specimens from skin sores (for cutaneous leishmaniasis) were collected and processed for PCR. The laboratory used for diagnosis PCR primers that target a conserved region common to *Leishmania* species [41]. Specific primers for determining the various complexes found in Central America (for example, the *L. mexicana* complex versus visceral Leishmaniasis strain *L. infantum*) are not necessary for diagnosis but may be useful for public health surveillance in countries where multiple *Leishmania* species co-exist [41, 42].

4.1.3.2. Case-based example: A patient presents with fever, weight loss, enlargement (swelling) of the spleen and liver, and abnormal blood tests such as low blood counts, including a low red blood cell

count (anemia), a low white blood cell count (leukopenia), and a low platelet count (thrombocytopenia). The healthcare provider performs a physical examination and determines that the patient traveled to Cyprus. The travel history was communicated to the laboratory on the requisition. A bone marrow is examined for the diagnosis of visceral leishmaniasis. Detection of *Leishmania* species is performed with genus-specific primers [42]. In this situation, the laboratory could also use primers validated for the identification of *Leishmania* species causing visceral leishmaniasis found in Europe such as *L. donovani* and *L. infantum*. In both these cases, travel history facilitated test interpretation and methodology for the diagnosis of cutaneous and visceral leishmaniasis [41, 43].

4.2. *Oncology: Clinically relevant information and the detailed description of an analyte on requisition*

4.2.1 Background: Human Papillomavirus (HPV) screening for cervical cancer is typically performed on patients older than 25 years. The requisition can indicate the age of the patient.

4.2.1.1. Case-based example: A 20 year old woman receives a pelvic examination and a cytology sample is collected for Papanicolaou test and HPV analysis. The laboratory accepts the sample for cytology but contacts the healthcare provider regarding the HPV test. The laboratory representative explains that HPV testing on a 20 year old patient does not follow the guidelines stated in the testing directory. Because the HPV test is used to identify risk for developing cervical cancer, testing of patients younger than 24 years old with a normal cytology sample is not warranted due to the high false positive rate [44, 45]. The provider requests that the HPV testing should be delayed until the cytology results are available.

4.2.2. Background: Testing for anaplastic lymphoma kinase (*ALK*) and c-ros oncogene 1(*ROS1*) gene fusions in lung cancer patients helps direct treatment. A variety of fusion partners were documented for these two genes. In the requisition form, specific gene fusions are listed.

4.2.2.1. Case-based example: A lung biopsy is submitted to the laboratory for *ALK* and *ROS1* gene fusion testing. The *ALK* fusion but not *ROS1* fusion was detected in the FFPE specimen by fluorescent *in situ* hybridization (FISH). The clinician requested consultation from the laboratory client services department since the RT qPCR test did not detect an *ALK* rearrangement. The clinician was educated on the limitations of both tests and referred back to the test directory and the requisition form. The test directory stated that the RT qPCR test usually detects only the most frequent *ALK* fusions and hence may not have included the rare fusion event detected by FISH [46].

4.3. *Inherited Diseases: Identifying the patient's ancestry and purpose of the test, ancillary information on the request form*

4.3.1. Background: Although the terms "race" and "ethnicity" are often on requisition forms, ISO 15189 recommends describing or identifying the patient's ancestry where relevant. As stated earlier, the healthcare provider should be educated on the limitations of the laboratory's testing methodology prior to sample collection. Ethnicity is considered a cultural behavior and self identifying race has been

shown to be problematic. The geographical origin of a patient may be useful for selecting appropriate pathogenic variants.

4.3.1.1. Case-based example: The laboratory received a request for inherited Creutzfeldt Jakob disease with no comment on ancestry, without preliminary screening of cerebrospinal fluid (CSF) biomarkers, and with a description of slow progressing dementia [47, 48]. The laboratory contacted the healthcare provider and determined that the patient was French and from the southeast part of France. The laboratory proceeded with the test using High Resolution melting (HRM) analysis and the result was positive for the variant. Thus, results were provided within three days to the requesting physician. This scenario represents appropriate test utilization and method selection by the laboratory. The rationale is as follows: the PRNP p.E200K variant, is associated to inherited Creutzfeldt Jakob disease, is frequently found in patients from the Ain (France) due to a founder effect [49]. Without knowledge of the patient's ancestry, additional and unnecessary testing may have been performed. Such unnecessary tests include biochemical analysis of the CSF to confirm the clinical utility of the test in absence of familial history and Sanger sequencing, which has a longer TAT than HRM.

4.3.1.2. Case-based example: The laboratory received a request for acute intermittent porphyria. Acute intermittent porphyria is a metabolic inherited disease affecting heme biosynthesis. Over 300 gene variants have been associated with acute intermittent porphyria. The request form stated that the patient was Caucasian and, in the comment section, that the patient was from Rotterdam, The Netherlands. The laboratory accepted the specimen and performed initial screening for the p.R116W variant in hydroxymethylbilane synthase gene (*HMBS*). Most mutations are private; however, the p.R116W mutation in *HMBS* is likely a founder mutation in the Dutch but not in the Venezuelan population [50, 51]. By stating the ancestry of the patient on the requisition form, the most appropriate test was performed.

4.3.2. Background: The purpose of the genetic test should be stated on the request form to ensure appropriate test selection and interpretation. It is not always sufficient to specify the gene name because some genes may be associated with multiple effects.

4.3.2.1. Case-based example: A blood sample was submitted to the laboratory for 5,10-methylenetetrahydrofolate reductase (*MTHFR*) variant analysis. The *MTHFR* is a key enzyme for intracellular folate homeostasis and metabolism. Variant analysis for p.Ala222Val and p.Glu429Ala was performed and identified a heterozygous variant for p.Ala222Val. Results were sent to the physician with the following conclusion: "The patient presents no particular risk factor for hyperhomocysteinemia and no related risk of thrombophilia [52, 53]." The healthcare provider contacted the laboratory expressing concern that the laboratory test interpretation lacked relevant information for the management of the patient recently diagnosed with acute leukemia. The healthcare provider had requested the *MTHFR* variant analysis to determine the patient's risk for having a toxic reaction to methotrexate. Certain *MTHFR* variants can be used to predict the susceptibility to antifolate and fluoropyrimidine agents [54]. In this situation, a statement in the request form could guide test interpretation.

4.3.2.2. Case-based example: A blood sample is submitted to the laboratory for the *UGT1A1* variant coding for UDP glucuronyltransferase 1 family, polypeptide A1. UGT 1 A1 is a transferase associated with conjugation of both endo- and xenobiotics that facilitates their elimination. *UGT1A1* variants are associated with inherited syndromes (specifically, Crigler Najjar and Gilbert syndromes) or with Irinotecan toxicity (specifically, a variant within the promoter also known as *28) [55, 56]. The test offered by the laboratory detects the *28 the promoter polymorphism but not the variants associated with the inherited disease. In this scenario, the patient is suspected of suffering from Gilbert syndromes, and is not being treated with irinotecan. Because the test request stated the name of the gene without providing the purpose of the test, an incorrect test was performed. This case illustrates the need for appropriate information on the requisition form so that the laboratory will be aware if the available test will adequately address the patient's needs. In the case that the testing methodology does not address the clinical need, a representative may contact the health care provider or redirect the sample to an outside laboratory.

4.4. Pharmacogenomics: Ancestry and clinical decision making

4.4.1. Background. Genome-wide association studies revealed that certain interleukin-28B (*IL28B*) polymorphisms are strongly associated with responses to pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy in patients with chronic hepatitis C (HCV genotype 1)[57]. There are three *IL28B* genotypes at rs8099917: CC, CT, and TT. People with the CC genotype have a stronger immune response to HCV infection than people with the CT or TT genotypes (*non-CC genotypes*). This immune response makes people with CC genotype more likely to clear HCV without treatment (spontaneous viral clearance), within months of becoming infected. People with CC genotype are two to three times more likely to be cured by PEG-IFN and RBV. *IL28B* genotypes also affect treatment efficacy in chronic infection with other HCV genotypes, inflammatory status, progression of fibrosis and adverse clinical outcomes in chronic hepatitis C. Ethnicity is also a factor in treatment outcome. The proportion of African American patients achieving sustained virologic response (SVR) on treatment with PEG-IFN/RBV is lower than Caucasian patients, indicating that host genetic factors can be an important determinant of treatment outcome [58].

4.4.1.1. Case-based example: A patient with chronic hepatitis C caused by HCV genotype 1 submits a sample for *IL28B* genotyping. The requisition states that the patient is Caucasian and has no history of HCV therapy. The test can provide the following information to the clinician. *IL28B* genotypes are strongly associated with PEG-IFN/RBV treatment efficacy in patients infected with HCV genotype 1 or 4 and to a lesser degree with other HCV genotypes. *IL28B* genotyping is also useful for pretreatment prediction of the outcome of direct-acting antivirals (DAAs) plus PEG-IFN/RBV therapy, especially in treatment-naïve patients. Future more aggressive treatments, such as quadruple therapy or potent DAAs combinations might obscure the influence of *IL28B*, but *IL28B* genotyping will remain useful for making decisions on suitable regimens and treatment duration in patients in the forthcoming era of DAAs [59].

5. Informed Consent

5.1. *Variables associated with written, signed or implied informed consent*

Consent (specifically, written and signed or implied) is required for all medical tests and procedures. For molecular tests, the degree of diligence/documentation in obtaining consent can vary widely depending on the test and on ethical and technical complexities (for example, implications for genetic relatives), and the geographical region involved. For most microbiological tests, the requirement for consent is similar to non-molecular testing since the molecular test is not being used to examine the patient's genetic composition. Although for some sexually transmitted infections, consent may be required. For inherited and genetic disorders, the broad consensus is that a higher level of diligence is required. Differences on the stringency of the need to obtain informed consent may vary on the basis of regional/national perspectives. Nevertheless, the consent should be informed so that the patient knows what they as the patient are agreeing to, including what is being tested for, why, and the potential outcomes including diagnostic accuracy, treatment options, implications for family members and how such testing affects insurance policies. In many instances, particularly when the test will generate a predictive result, genetic counseling is required as part of the consent process (Figure 1).

5.1.1. Background: Legislative, accreditation and other mandatory requirements

Institute and laboratory directors must ensure they comply with all applicable national and regional laws, and national/local requirements pertaining to delivery of molecular testing, for example as may be required by accrediting organizations requirements. Substantial differences in requirements may exist and may vary according to location/region. In numerous instances, national research councils make recommendations on the requirement for written consent and professional genetic counseling.

5.1.1.1. Case-based example: A patient with a history of venous thrombosis consults with her healthcare provider in Germany. The healthcare provider in Germany must obtain informed consent prior to testing for the genetic Factor V Leiden variant (FVL) associated with thrombosis [60]. Testing for all Factor V Leiden requires informed and written consent in Germany. Such consent includes sufficient information on the extent on the planned genetic testing, as well as the possibility to destroy the performed test result (also known as, the right of not-knowing [Figure2]). This is not necessarily required elsewhere[61]. For instance in Australia, Australia's National Pathology Accreditation Advisory Council (NPAAC) stratifies genetic testing into categories. Specifically, testing for Factor V Leiden and all genetic tests are grouped into two levels requiring different levels of informed consent. Level 1 tests are generally simple genetic tests, as applied in uncomplicated ethical scenarios used for diagnostic purposes (for example, FVL in a patient with thrombosis or an established family history) or for screening purposes, and do not usually require formal written consent. By contrast, application of the same test in an asymptomatic individual would be classified as a Level 2, hence requiring consent [62, 63].

5.1.1.2. Case-based example: A patient in New York state submits a sample for genetic testing. Three months later, the healthcare provider requests additional testing on the previous DNA sample. Because the patient was initially tested in New York State, no additional testing could be performed because the

sample was required to be disposed of within 60 days. This scenario illustrates regional differences in consent. A 2009 CDC report lists numerous states in the US that had legislated a requirement for formal consent) [64]. Examination of this document shows numerous common elements (specifically, purpose of the test, who the result will be disclosed to, etc.), but there are also significant differences. Of the 8 states listed, only one, New York, gave strict guidelines on how long the sample could be kept once the test had been completed (60 days).

5.1.1.3. Case-based example: A healthcare provider submits a request for a genetic test for a patient. The test may or may not be accepted by the laboratory if the requisition requires an indication that the healthcare provider has obtained informed consent. Some request forms require an indication by the healthcare provider that written consent has been obtained. Such documentation is good practice and is required in some jurisdictions (for example, Level 2 tests in Australia), even if accreditation or other requirements (for example, the Clinical Laboratory Improvement Act [CLIA]) may not require it [64].

6. Laboratory Handling, Monitoring and Storage of the Primary Sample Prior to and following Testing

Following sample collection and arrival in the laboratory, the laboratory assumes various responsibilities for the sample. The laboratory should inspect the samples upon reception into the laboratory. The inspection may include confirming that the samples have been appropriately and unequivocally labeled and verifying that the collection material is within the approved expiration date. The laboratory can also monitor the specimen's characteristics specifically the type of additive in the sample collection (such as heparin), whether the specimen is fixed versus not fixed, and clinical information such as leukopenia or if a recent blood transfusion is mentioned in the requisition. The laboratory may, if possible, monitor various aspects of transportation such as temperature and the time taken for transport and delivery. The following case-based examples illustrate the potential problems following sample intake into the laboratory and the actions that a laboratory might take to insure quality for molecular diagnostic testing.

6.1. *Infectious Disease: Patient identification on container and transportation, sample handling/storage prior to testing*

6.1.1. Background: Pulmonary HSV infections cause morbidity and mortality in immunocompromised and/or patients with pulmonary disease such as lung cancer. Bronchial washings are collected in patients suspected of having pulmonary HSV [65, 66]. Respiratory syncytial virus (RSV) is an RNA virus and a significant cause of pediatric respiratory tract infections [67]. Differentiation between RSV and potential bacterial infections determines which therapeutic approach the healthcare provide should implement.

6.1.1.1. Case-based example: The laboratory received bronchial washing contaminated by blood in an unlabelled tube without clinical information requesting screening HSV-1 and HSV-2 tests. The laboratory rejected the sample for testing on all stated criteria, since blood interferes with the PCR based method used. Additionally, the sample was not appropriately labeled upon collection.

6.1.1.2. Case-based example: A young patient with fever and suspected a viral respiratory disease is positive for the RSV antigen based on analysis by a point of care (POCT) device. To confirm the diagnosis a swab (with media) is taken and shipped to the laboratory at room temperature. The transport was delayed by more than 36 hours. RSV PCR testing by the laboratory gave negative result due to sample/RSV degradation [68]. The laboratory should consider changing specimen collection media to improve patient care and/or affect logistical changes that will insure prompt delivery of the sample.

6.1.2. Background. In some situations, samples may be stored prior to testing if the integrity of the sample is not compromised by storage. Some reasons for storing samples include testing samples at the optimal batch size to decrease cost, implementing testing on certain days to comply with production schedules and/or offering testing when adequate staffing is available.

6.1.2.1. Case-based example: A laboratory carries out validation comparing the effect of freezing/thawing on the integrity of samples for viral load estimation. The laboratory noted the frequent freeze/thaw cycles decreased viral loads when compared to samples stored at room temperature. The outcome of this validation ensured that the laboratory was properly storing samples (specifically, refrigeration) for viral load testing [69].

6.2. *Oncology: Types of samples labeling and processing individual biopsies*

6.2.1. Background: The septin family of genes include *SEPT9*. Septins are involved with cell cycle control. *SEPT9* may be a tumor suppressor [70].

6.2.1.1. Case-based example. Screening for *SEPT9* methylation status was requested on a FFPE section delivered to the laboratory from a patient with colorectal carcinoma. The laboratory requested information documenting the histology of the tumor, the source and procedures used for section preparation. The histological information satisfied the pathologist's requirements for molecular testing but the section was prepared from a FFPE block 2 months ago. The test is validated for tumor DNA shed in the bloodstream and the DNA isolated from submitted FFPE sections may have acquired modifications (specifically, fragmentation and crosslinking) thus compromising the test result [71, 72]. Accordingly, the sample was rejected for examination.

6.2.2. Background: Cutaneous T-cell lymphoma may present with multiple lesions requiring multiple biopsies. Each biopsy should be separately labeled and the location of the biopsy noted. Each biopsy may then be processed separately and analyzed for T cell receptor gamma gene rearrangements.

6.2.2.1. Case based example: Multiple biopsies are collected for T cell gamma rearrangement analysis. Clonal analysis was performed on each biopsy. By labeling and processing each biopsy separately, the laboratory was able to determine if any T cell clones were shared between the biopsies from separate physical locations [73].

6.2.3. Background: As discussed in 3.2.3, RT qPCR is one method for therapeutic monitoring of CML. Because the method requires isolation of mRNA, the stability of the sample is critical.

6.2.3.1. Case-based example: The laboratory received EDTA anticoagulated peripheral blood from a CML patient and an additional blood sample tube containing a nucleic acid stabilization component, which is particularly important for mRNA analysis. Both blood samples were left in the collection station overnight. The EDTA sample was rejected for testing since the pre-examination phase did not adhere to the laboratory service manual; however, the sample containing stabilization media was accepted for testing [74].

6.3. *Inherited Disease: Additives in sample and minimal number of cells*

6.3.1. Background: Blood collections for molecular testing should avoid heparin containing tubes as heparin can interfere in molecular assays [75, 76]. In contrast to receiving samples that have inhibitors, adequate samples or volumes are critical in obtaining quality results.

6.3.1.1. Case-based example: A young patient is to be investigated for a number of genetically determined heart diseases. The patient sample was collected using heparin as an anticoagulant, and resulting in PCR failure. Spectrophotometric analysis of the extracted DNA demonstrated sufficient DNA. Some laboratories may accept heparin tubes but in doing so these laboratories must add procedures to the processing step to reverse the inhibition caused by heparin [77].

6.3.1.2. Case-based example: The laboratory provided information on the minimal white blood cell count needed for a test. The healthcare provider notified the laboratory that they would submit a sample from a patient with severe leukopenia. Because the laboratory was made aware of the exceptional low level of DNA expected from the collection, a DNA extraction method capable of improving the DNA yield was used prior to testing [78].

6.4. *Inherited Diseases: Documenting time of receipt of sample*

6.4.1. Background: NIPT permits genetic testing on samples collected from peripheral blood rather than through amniocentesis [79]. The noninvasive approach decreases risk of fetal miscarriage.

6.4.1.1. Case-based example: A blood sample (collected with EDTA anticoagulant) used for DNA analysis was collected, stored at room temperature and sent to the laboratory for processing within six hours. The time that the sample was received was electronically recorded and logged into the laboratory information system. This case illustrates the importance of sample tracking processing including confirmation of appropriate sample collection tube and timeliness of sample processing. Had the sample been processed after 6 hours, damage to blood cells can result in the release of maternal DNA into plasma leading to the dilution of the target DNA for NIPT analysis and lowering of the percentage of fetal DNA in the sample. If the laboratory cannot provide logistical support for prompt sample processing, alternative blood collection tubes for cell-free DNA analysis (for example, Streck tubes [Omaha NE]), with the capability of extending the time to seven days may need to be considered to insure patient care [80, 81].

6.4. *General Considerations for Nucleic Acids Quality: Long term storage of patient sample and calibrators*

6.4.1. Background: Purified nucleic acids from patients need to be maintained in the event that the sample requires retesting. Additionally, qPCR may use nucleic acid calibrators to quantify the amount of targeted PCR product.

6.4.1.1. Case-based example: Patient samples were stored in molecular grade water. The sample was required for retesting two years later and the sample had degraded significantly during this period of time. The purified water was determined to be acidic and may have caused DNA depurination.

6.4.1.2. Case-based example: As part of the competency training, different technologists performed the qPCR using the stored calibrators in the laboratory. The calibrators were stored in water while other calibrators were stored in 50% glycerol. Comparison of results demonstrated that the calibrators stored in 50% glycerol yield higher amounts of DNA compared with calibrators stored in water [82].

6.4.1.3. Case-based example: Samples were stored in a frost-free freezer in a standard rack. Frost free freezers cycle between freezing and non-freezing temperatures so that frost does not build up in the freezer. The samples that were stored in the standard rack degraded at a higher rate than those maintained in the separate insulated container within the freezer. It was determined that the repeated formation of ice crystals were shearing DNA in the rack but this was not so for the sample stored in the insulated container [83].

7. Discussion

Standardization of patient care occurs when laboratories follow similar guidelines. Many guidelines are available for molecular diagnostics laboratories. An international panel of molecular diagnostics experts identified that ISO 15189 was the most frequently used guidance. The ISO 15189 standard is general in its content for laboratory management and technical aspects of testing. In this review, the IFCC-MD selected cases from different subspecialties of molecular diagnostics for the pre-examination phase of the testing. The importance of controlling and structuring the pre-examination phase (also known as pre-analytical phase) for molecular diagnostics and of clarifying ambiguities is illustrated by two international efforts: the first is to adapt interpretation of ISO 15189 to molecular genetics by CLSI (specifically, MM20 guideline) and, the second is the European Committee for Standardization (CEN) draft document for pre-examination phase [81, 84]. The case-based examples are not meant to illustrate every ISO 15189 pre-examination requirement but are meant to underscore the value of selected recommendations. Additionally, the clinical vignettes presented here provide a level of interpretation for readers. The pre-examination phase of the laboratory testing can be particularly challenging as many aspects are beyond the direct control of the laboratory. Communication between the laboratory and the healthcare provider is a recurring theme. Specifically, the cases illustrate the value of direct and indirect communication between the laboratory and the healthcare provider. In some cases such as TAT determination, the laboratory and the healthcare provider may need to collaborate to meet the needs of the patient. Setting expectations for the need for information on request forms, sample collection, sample labeling, rejection criteria, transportation and storage improves the clinical value of diagnostic and prognostic information to the healthcare provider.

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Background: Multiple organizations produce guidance documents that provide opportunities to harmonize quality practices for diagnostic testing. The International Organization for Standardization ISO 15189 standard addresses requirements for quality in management and technical aspects of the clinical laboratory. One technical aspect addresses the complexities of the pre-examination phase prior to diagnostic testing.

Methods: The Committee for Molecular Diagnostics of the International Federation for Clinical Chemistry and Laboratory Medicine (also known as, IFCC C-MD) conducted a survey of international molecular laboratories and determined ISO 15189 to be the most referenced guidance document. In this review, the IFCC C-MD provides case-based examples illustrating the value of select pre-examination processes as these processes relate to molecular diagnostic testing. Case-based examples in infectious disease, oncology, inherited disease and pharmacogenomics address the utility of: 1) providing information to patients and users, 2) designing requisition forms, 3) obtaining informed consent and 4) maintaining sample integrity prior to testing.

Conclusions: The pre-examination phase requires extensive and consistent communication between the laboratory, the healthcare provider and the end user. The clinical vignettes presented in this paper illustrate the value of applying select ISO 15189 recommendations for general laboratory to the more specialized area of Molecular Diagnostics.

Figure legends

Figure 1. Example of consent form for genetic tests. Used with permission from the Human Genetics Society of Australia (reproduced with permission).

Consent Form for Genomic Testing	
PROVISION OF INFORMATION TO PATIENT To be completed by Health Professional Step 1: Testing a panel of genes known to cause: _____ (specify condition) Step 2: Testing of all genes (Genomic Testing) Possible Outcomes of Genetic Testing: 1. An informative result means that a mutation has been found and that it explains the diagnosis. 2. If a mutation is not found, the result is uninformative. This may be because a mutation is present but could not be found using current technology. An uninformative result does not exclude the diagnosis. 3. Results of unknown significance: sometimes a variation in a gene is found but its meaning is unclear. In this situation, further testing of other family members may be required. The interpretation of a result with unknown significance may also alter as knowledge of genetics improves. I may be contacted if this occurs however the time frame for any additional results is not known. 4. Testing may reveal non-paternity or non-maternity of a presumed natural parent 5. Testing will not affect the ability to obtain health insurance but may affect applications for some types of life insurance. 6. Relevant clinical testing results will be given to me in person. 7. The information from genetic testing will be stored by the laboratory according to government regulations. 8. If I agree, the de-identified genetic information may be placed in databases and shared with other researchers. The chance of someone identifying me from these data is small. Results from research tests are rarely returned to participants, and there is no promise of benefit to me. 9. Testing is voluntary and it is possible to withdraw from the testing process at any stage without influencing my management /the management of my relatives. However, de-identified data that have already been shared with other researchers cannot be retrieved. PATIENT CONSENT To be Completed by the Patient/ Guardian 1. I consent to testing a panel of genes known to cause _____ ☐ YES ☐ NO 2. I consent to genomic testing, if (a) this initial testing does not identify a cause, or (b) my doctor advises that I should go directly to testing all genes at the same time. I am aware that there is a small chance that co-incidental findings about my/my child's health not related to the diagnosis in me/my child may be identified when genomic testing is carried out. The significance of co-incidental findings will be assessed confidentially by an independent expert committee. I will be informed of these co-incidental findings only if the committee, in consultation with my doctor or genetic counsellor, assess that these findings could have a significant impact on my/my child's/my relatives' health. ☐ YES, I consent to genomic testing ☐ NO, I do not consent to genomic testing	3. Sharing the result with blood relatives and family members: • My test result may have implications for the health care of my blood relatives. If it does, I consent to my result being given to relevant blood relatives and health professionals involved in their care. ☐ Yes ☐ No, only the following people: _____ • In the event of my death, the test results may be given to: Name: _____ Relationship: _____ Contact Details: _____ Name: _____ Relationship: _____ Contact Details: _____ 4. I agree that my de-identified genetic data can be made available for research: ☐ YES ☐ NO I request and consent to the test described above. I have read the genomic testing information sheet and understand the potential benefits, limitations and consequences involved in the testing and storage of this sample and information. I allow my/my child's de-identified data to undergo genetic research activities that have been approved by a recognized ethics committee. I have had the opportunity to ask additional questions and I am satisfied with the explanations. I understand that genetic counseling will be available for myself and my family. Signature of Person being tested _____ Print Name _____ Signature of Parent / Next of Kin / Guardian _____ Print Name _____ Date _____ I, _____ Name of Health Professional and Designation _____ have informed this patient/parent/guardian as detailed above, about the nature, limitations, likely results and risks associated with the testing of genes. We have discussed the procedures and consequences of testing and the storage of patient data. Signature of Health Professional _____ Signature of Interpreter (if present) _____ Date _____

Figure 2. Example of a documentation form: Documentation of education / genetic counseling before / after genetic testing according Gendiagnostikgesetz (GenDG) from Germany documenting the performed education of the patient and the genetic counseling. Such consent forms typically include that the patient was informed that his consent may be withdrawn at any time without giving reasons and without any kind of disadvantages for him. The patient has the right not to know his results. Please note, that this is an example from Germany as a consequence from the German Genetic Diagnosis Act-GenDG and may be managed differently in other countries.

2. Confirmation of concluded clarifications and/or genetic counselling (delete as applicable)	
The disease / disorder / diagnosis..... its genetic background, prophylaxis/prevention/treatment possibilities as well as purpose, nature, scope and information value of a possible genetic diagnostic including the risks attached to the taking of samples and/or the tests have been made sufficiently clear to me and counselling in these matters was adequate.	☐ yes ☐ no
I was advised that I am entitled to withdraw my agreement in whole or in part without giving reasons and without any penalties resulting from this withdrawal. I know that I have the right not to be informed of the examination result(s) (right not to know). I was advised that I can stop the commenced examination procedure until the communication of the results at any time and that I can demand the destruction of all of my examination material including all components extracted from it as well as of all results and findings obtained so far.	☐ yes ☐ no
I agree to the findings of the genetic testing being sent to: - My general practitioner: Dr. - My gynaecologist: Dr. - Dr.: - Dr.:	☐ yes ☐ no
Place, date of clarifications/genetic counselling before the examination:	Patient's signature or legal representative:
Place, date of genetic counselling after presentation of results:	Patient's signature or legal representative:

3. Documentation by the consultant clarifying/counselling according to GenDG [Genetic Diagnosis Act] (use additional sheet if necessary)	
Anamnestic characteristics:	
Documentation of clarification/counselling contents before examination: ☐ genetic background ☐ risk of repetition ☐ prophylaxis/prevention/treatment possibilities ☐ purpose, nature, scope and information value of examination ☐ risks attached to the taking of samples and/or the tests ☐ Other:	Documentation of clarification/counselling contents after presentation of results: ☐ genetic background ☐ risk of repetition ☐ prophylaxis/prevention/treatment possibilities ☐ purpose, nature, scope and information value of examination ☐ risks attached to the taking of samples and/or the tests ☐ Other:
Procedure:	Procedure:
Place, date:	Place, date:
Signature of the physician responsible according to GenDG	Signature of the physician responsible according to GenDG

Highlights: Pre-Examination Factors Affecting Molecular Diagnostic Test Results and Interpretation: a Case-Based Approach

- Select ISO 15189 recommendations for the general laboratory are interpreted in the context of clinical vignettes for molecular diagnostic tests (specifically infectious disease, inherited disease, oncology, and pharmacogenomics).
- The pre-examination phase for molecular diagnostic tests requires direct and indirect communication with healthcare providers.
- The consent forms needed for molecular diagnostics vary according to geographical location and test complexity
- Standardization of the pre-examination phase improves test utilization, specimen integrity and the quality of test results