Wednesday, August 1, 2018

Poster Session: 9:30 AM - 5:00 PM

Molecular Pathology/Probes

B-266

Detection of possible new cytogenetic alteration by arrayCGH related to dysmorphia in the hands

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Background: Comparative Genomic Hybridization Arrays (CGHarrays) are technologies that allow detecting losses or gains of genetic material and unbalanced rearrangements throughout the genome with high sensitivity, speed and resolution. **Methods:** A 13 year old patient with a possible dysmorphic syndrome is studied for presenting disorder of the joint movement of the fingers of both hands that hinder movement. As a family history he emphasizes that his father has similar alterations in the fingers. The arrayCGH is carried out with the PerkinElmer platform which has an average resolution of 20kb and of 10kb in the regions of interest. The Genoglyphix software is used to interpret the results. **Results:** A deletion of 1.98 Megabases is detected in the chromosomal region 1q41 The study is also carried out in his father for presenting also the pathology detecting the same alteration found in his son. **Conclusion:** The deletion found has not been associated to date pathology or polymorphism, so it could be considered as of clinical significance uncertain, but when detected also in the parent with a high probability could be cataloged this alteration as the Cause of pathology. With this technique can be revealed alterations that can be cause of mental retardation, intellectual disability, dismorfias and/or malformations, which represent a major public health problem. Thanks to the arrayCGH, the proportion of cases whose etiology is unknown can be minimised.

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A case of 4p deletion syndrome inherited from an affected parent without phenotypic features

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Background: Partial deletions of the short arm of chromosome 4 is associated with a clinically recognizable syndrome. Wolf-Hirschhorn syndrome (WHS) is a rare chromosomal disorder caused by terminal deletion of the short arm of chromosome 4. About 50%-60% of individuals with WHS have a *de novo* deletion and about 40%-45% have an unbalanced translocation with a deletion of 4p. These unbalanced translocations may be *de novo* or inherited from a parent with a balanced rearrangement. The clinical picture includes facial anomalies, growth retardation, mental retardation, seizures and defects in the brain, heart and palate. It is reported that the sizes of 4p deletions are variable and the clinical findings are different from classic WHS according to the deleted portion. The critical region for WHS is located on the terminal part of the chromosome 4p, with a length of approximately 1-5 Mb. The severity of the clinical presentation is variable depending on the haploinsufficiency of genes in a deleted region. **Objective:** To report a case of a maternally inherited 4p deletion from asymptomatic woman analyzed by G-banding karyotype by array-based comparative genomic hybridization (array-CGH). **Case report:** We report four-year-old boy with severe hypotonia, hypospadias, psychomotor development delay, hypertelorism, cardiac anomalies, seizures and defects in the brain. He was born to nonconsanguineous and asymptomatic parents, with no history of obortions. G-band chromosome analysis revealed the karyotype 46, XY, del(4)(p16). His father had a normal karyotype (46,XY), but the maternal chromosomal analysis revealed the 46,XX,add(4)(p15.2) karyotype. The proband's array-CGH analysis revealed an 8.6 Mb pathogenic deletion in the chromosome 4p16.3p16.1 [arr[hg19] 4p16.3p16.1(85,743-8,702,376)x1] encompassing more than 80 genes, including the WHS critical region. Maternal CGH array analysis revealed an interstitial 5.5 Mb deletion in the chromosome 4p16.3p16.1. This deletion includes several OMIM genes such as HTT, DOK7, ADRA2C, EVC, EVC2, but these genes alone were not related to any phenotype, except for the MSX1 and WFS1 genes. The WFS1 gene is relates to Wolfram Syndrome and non-syndromic sensorineural hearing loss, whereas the haploinsufficiency of the MSX1 gene is associated with selective dental agenesis. Thus, a smaller deletion was detected in this case, excluding the WHS critical region. The recurrence of WHS in the child was not due to a parental balanced translocation, but it was due to a different mechanism, the meiotic amplification of a smaller deletion present in the mother, most likely. This case highlights the importance of an integrated approach to cytogenetic analysis and array-CGH analysis.

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Expression of Tim-3 in PBMCs and Placental Tissue in Unexplained Recurrent Spontaneous Abortion

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Background: The expression of T-cell immunoglobulin domain, mucin domain-3 (Tim-3) in unexplained recurrent spontaneous abortion (URSA) was investigated. **Methods:** im-3 mRNA expression in peripheral blood mononuclear cells (PBMCs) of URSA and control groups was assayed by fluorescent quantitative real-time polymerase chain reaction. Tim-3 protein expression intensity and localization in placental villi and uterine decidua were determined using immunohistochemical assay. **Results:** Tim-3 mRNA expression in PBMCs was significantly higher in URSA than in normal controls (1.32 ± 0.13 vs. 1.20 ± 0.06, $P < 0.01$). Tim-3 was expressed in placental tissue from both URSA patients and normal pregnant females (controls); however, the expression intensity was higher in the URSA group ($P < 0.05$). **Conclusion:** Increased Tim-3 expression in PBMCs may affect maternal-fetal immune tolerance and facilitate pathogenesis of URSA.

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distribution of the genotype of interleukin-28b sr12979860 in patients with chronic hepatitis c studied in our hospital area

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Background: According to data from the World Health Organization, infection with the hepatitis C virus represents a serious global health problem, since it is estimated that it affects approximately 180 million people, of which 70-80% will develop an infection chronicle. In these patients with chronic infection, the polymorphism rs12979860 of IL28B has been described as an important predictor of response to treatment with pegylated interferon alfa (INF alpha) and ribavirin. Thus, patients carrying the CC polymorphism have percentages higher than 80% sustained viral response (SVR), while CT and TT patients have SVR rates of less than 50%. The aim of this study is to determine the distribution and frequency of the different polymorphisms of IL28B rs 12979860 in patients with chronic HCV infection studied in our sanitary area. **Methods:** A descriptive, retrospective study was conducted to determine the distribution of IL28B rs 12979860 in patients with chronic HCV studied in our health area for a period of 6 years (January 2011 to January 2017). The IL28 polymorphism determination was performed by real-time polymerase chain reaction (PCR), using specific fluorescent probes for the C and T alleles of SPN rs 12979860. **Results:** From January 2011 to January 2017 2465 patients were analyzed. Males represented 71% of the studied population with an average age of 54 years with a range of 29 to 79. Women accounted for 29% with an average age of 51 years and a range of 31 to 83 years. The analysis of the distribution of IL28B polymorphism results showed that 36.31% (895/2465) were homozygous CC, 49.86% (1229/2465) were heterozygous CT, and 13.83% (341/2465) were homozygous TT. **Conclusions:** -There is a high percentage (63.69%) of patients with CT and TT polymorphism that are associated with low rates of sustained viral response to treatment with IFN alpha and rivabirine. -As in the literature consulted, the CC genotype was found in 36.31% of these patients. -The typing of the IL28B polymorphism allows evaluating the probability of response to INF plus ribavirin in patients with chronic HCV allowing the adjustment thereof over time by following the individual requirements increasing it in individuals with CC genotype and looking for new therapies in the CT and TT genotypes.

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ABL1 transcripts levels in peripheral blood mononuclear cells of healthy individuals: comparison of three automated nucleic acid extraction instruments.

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Background: The ABL1 mRNA is widely used as the reference gene for quantification of leukemic fusion transcripts, such as BCR-ABL and PML-RARA. The ABL1 quantification is used to ensure the analytical assay sensitivity, which is essential for the minimal residual disease monitoring along and after therapy. A sensitive BCR-ABL and PML-RARA RT-qPCR process should quantify 10^5 copies of ABL transcripts in every reaction. Here, we compared three automated systems for nucleic acids (NA) extraction from peripheral blood mononuclear cells (PBMC) to evaluate the ABL1 levels returned by each system. **Methods**: PBMC of healthy volunteers (60 women and 26 men, average age 42.21±17.59 years) was isolated from EDTA-whole blood samples (4 mL, n=86). Residual red blood cells were removed using ammonium chloride-based buffer (BD Biosciences). PBMC was resuspended in the initial sample volume required for each equipment. PBMC total nucleic acids (or RNA) were extracted on QIAcube (Qiagen) using RNeasy kit (PBMC resuspended in 600 uL, RNA elution in 100 uL) (n=24); on MagNA Pure 96 (Roche) using DNA/Viral large volume kit (PBMC resuspended in 1mL, NA elution in 100uL) (n=32); or on easyMAG (Biomerieux) (PBMC resuspended in 1mL, NA elution in 65 uL) (n=30). ABL1 mRNA was amplified by one-step RT-qPCR using Europe Against Cancer primers/probes and QuantiNova probe master mix (with DNase) (Qiagen) on Light Cycler 480 II or 480z (Roche). Quantification standard curves were constructed with ssDNA oligos corresponding to the ABL1 primers molecular target. Medians ABL1 copy number per reaction were compared using Kruskal-Wallis test followed by Dunn's multiple comparisons test. The percentage of samples with higher than 10^5 copies per reaction of ABL1 transcripts were compared by Chi-squared test followed by Marascuilo procedure (Multiple comparisons). **Results**: The median (Max-Min) of ABL1 copies per reaction was $7.5x10^4$ ($1.5x10^5$-$2.4x10^4$) for QIAcube, $1.3x10^5$ ($1.7x10^5$-$5.8x10^4$) for MagNAPure and $1.1x10^5$ ($2.6x10^5$-$7.6x10^3$) for easyMAG. The median copy number obtained using easyMAG and MagNAPure were similar (p=0.40). However, both were different from QIAcube results (p=0.0001 and p=0.0011, respectively). The percentage of samples that achieved 10^5 copies of ABL transcripts per reaction was 25% for Qiacube (p<0.0001 *versus* easyMAG and p=0.0083 *versus* MagNAPure), 85% for MagNAPure (P=0.27 *versus* easyMag) and 97% for easyMAG. **Conclusion**: EasyMAG and MagNAPure extracted a higher median copy number of ABL transcripts per reaction compared to QIAcube. Additionally, a higher percentage of samples reached the limit of 10^5 copies of ABL transcripts using the prior instruments. These results suggest that easyMAG and MagNAPure can consistently provide better sensitivity for the assessment for the minimal residual disease of leukemic fusion transcripts. The limitation of this study is its small number of tested samples.

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Frequency distribution of p210, p190, and p230 fusions transcripts on a BCR-ABL1 laboratory routine

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Background: Chronic myeloid leukemia (CML) is a disease of hematopoietic stem cells present in 90% of CML patients that originate from the reciprocal translocation of ABL1 sequences from chromosome 9 to BCR sequences on chromosome 22. Around 95% of the CML patients are reported with e14a2/e13a2 (p210) BCR-ABL1. A very few patients had the BCR-ABL1 fusion e1a2 (p190). The BCR-ABL1 fusion e19a3 (p230) is even rarer. Co-expression of p210 and p190 in patients with classical CML or ALL have been described. Recently our group validated a simplified workflow for simultaneous detection/quantification of BCR-ABL1 e14a2/e13a2 (p210), e1a2 (p190), and e19a2 (p230) fusions by one-step RT-qPCR using ΔΔCq method. Here, we to describe the assay's frequency of results from August 15, 2017, to February 19, 2018. **Methods**: EDTA-whole blood (8 mL) or bone marrow samples (4mL) of 126 individuals (57 female, 69 male) were processed and tested for BCR-ABL1 e14a2/e13a2 (p210), e1a2 (p190) and 19a2 (p230) mRNAs. ABL1 was used as reference gene. Nucleic acids were extracted by using Magna 96 (Roche). We used the one-step RT-qPCR QuantiNova Probe master mix (Qiagen) on the routine assays. Primers and probes used were described by Gabert et al. 2003 (EAC) and by Pane et al. 1996. BCR-ABL1 and ABL RNAs were co-amplified in a multiplex one-step RT-qPCR reaction performed in the Roche LightCycler Z480 or 480II. P210 was calibrated to International Scale (IS) with a calibrator panel (Asuragen). Each fusion reaction contained Three levels of quality control material were used in each fusion reaction. The molecular response (MR) scoring used on follow-up patients was described by Cross et al. 2015. Descriptive statistics were determined for all the datasets. **Results**: BCR-ABL1 mRNAs assay was performed on 125 samples and 54 (43.2%) tested positive (26 males and 28 female). The median age of positive patients was 52 (range 8-82 years). Twenty positive patients (37.03%) had high expression levels of p210, 12 (22.22%) co-expressed p190 at low levels. One positive patient (1.85%) had higher expression levels of p230 and co-expressed p190 at a low level. Among the patients with low expression levels of BCR-ABL1 transcripts (<10%), 32 (59.25%) had p210, 1 (1.85%) p230 and 1 (1.85%) p190. Among the p210 with low levels, 9 (28.12%) were at MR1 (1-10%), 11 (34.37%) were at MR2(0.1-1%), 11 (34.37%) were at MR3 (0.01-0.1%), and 1 (3.12%) was at MR4 (0.001-0.01%). **Conclusion**: Co-expression of more than one BCR-ABL1 fusion was common in samples with high expression levels of p210 and p230, which is in agreement with literature data and may be explained by the alternative splicing. It is also important to point out that clinical laboratories usually do not test p230 BCR-ABL1 and we found the presence of two p230 positive patients and only one p190 positive patient, suggesting the importance of testing for these transcripts on the BCR-ABL1 fusions detection routine. The main drawback was the low number of samples tested.

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Whole-exome sequencing as first-tier testing approach for identification of the causal mutations in hereditary spherocytosis candidate genes and the use of non-sanger-based methods for validation of the findings

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Background: Hereditary spherocytosis (HS) is a common form of inherited hemolytic anemia characterized by hemolysis, jaundice, splenomegaly, and gallstones. The condition commonly exhibits an autosomal dominant pattern of inheritance. Causal mutations in at least five genes have been reported so far (SPTA1, SPTB, ANK1, SLC4A1, and EPB42). In this study, we aimed to investigate the five HS genes performing whole-exome sequencing (WES) to identify the causal mutations in a cohort of subjects with the condition. A secondary aim, we tested if non-sanger-based methods, such as ARMS-qPCR (for SNV) and capillary electrophoresis (for INDELs), can be used to validate the next-generation sequencing findings. **Methods:** The studied cohort comprises 16 patients (8 males) with HS diagnosis. There was one pair of siblings, one mother-daughter pair, one mother and two sons, and nine unrelated subjects. DNA was extracted from whole blood using MagNA Pure 96 (Roche), quantitated using Qubit (Thermofisher), and sized and qualified by gel electrophoresis. Targeted resequencing library was captured using SureSelect All exon V5-post kit (Agilent Technologies). Genomic libraries were quantified and validated using TapeStation (Agilent Technologies) and sequenced as paired-end 150 bp reads on the NextSeq 500 sequencer (Illumina). Sequence reads were aligned to human reference genome 19 using the BWA aligner. Duplicates were removed using Picard and variant calling was performed using the GATK Unified Genotyper (both from Broad Institute). SNV and INDELs were annotated using BaseSpace Variant Interpreter (Illumina). Variant lists were filtered based on having a minor allele frequency of less than 0.01 on population frequency databases [1000 Genome Project, NHLBI exome sequencing project and exome aggregation consortium (ExAc)]. Mutations that had read depth (coverage) less than 8, quality score less than 20, or resulted in synonymous amino acid changes were excluded. Sanger sequencing and ARMS-qPCR or capillary electrophoresis were compared for the validation of NGS results. **Results:** Pathogenic mutations were identified in 14 out of 16 (87.5%) studied subjects (all in heterozygosis). Six patients had a mutation in the SPTB gene [p.(Arg1423Ter) occurring twice and p.(Gln804Ter) occurring three times, because the subjects were relatives], six patients had a mutation in the ANK1 gene [p.(Arg319Ter), p.(Gln1806Ter), p.(Glu101Ter), p.(Gly122Arg), c.4227+1G>A and c.5518-1G>A], one patient had a mutation in the SPTA1 gene [p.(Tyr2305Ter)]. All of them have not been previously described and appear to be novel findings. One patient had both SLC4A1 [p.(Trp848Arg)] and SPTA1 [p.(Glu110Val)] mutations, which were found in the ExAc population database at very low frequencies (MAF of 0.000015 and 0.00022, respectively). All identified mutations were confirmed by Sanger sequencing and by ARMS-qPCR or capillary electrophoresis. Two patients (siblings) had no mutation in the five HS genes according to the used filtering criteria and will proceed to all hereditary anemia genes sequence inspection (followed by WES inspection if none causal mutation is found). **Conclusions:** The use of whole-exome sequencing as the first-tier testing approach was effective in identifying the causal mutations of HS in the studied cohort (87.5%

of the volunteers had the causal mutation identified) and non-sanger-based methods could validate the NGS results.

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Agreement between UV absorbance, automated microfluidic electrophoresis and qPCR methods for DNA quantification

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Background: DNA quantification is a critical step for next-generation sequencing (NGS) aiming to investigate genomic phenomena (e.g., mutations in cancer and diseases). Accurate quantification of the DNA starting material ensures consistent and reproducible results from NGS library preparation. Here, we aim to compare three methods with different methodologies for NGS input DNA quantification. **Methods:** Genomic DNA from 28 volunteers was extracted from 200 ul of EDTA-whole blood using MagNA pure 96 DNA and Viral NA Small Volume Kit on MagNA pure 96 instrument (Roche Diagnostics) according to the manufacturer's specifications. One microliter of extracted DNA was quantified by three distinct methods: UV absorbance (NanoDrop, Thermo scientific), automated microfluidic electrophoresis (TapeStation using genomic DNA screen Tape, Agilent Technologies) and qPCR targeting a 65 bp single copy genomic sequence (absolute quantification of RNase P gene, in-house). Mean DNA concentrations obtained by each method were compared using repeated measures one-way ANOVA with Geisser-Greenhouse correction followed by Tukey multiple comparisons test. Pearson correlations coefficient and Bland-Altman analysis of the tested methods were calculated. **Results:** The DNA yield (mean ± sd) measured by UV absorbance, automated microfluidic electrophoresis and qPCR were 50.71 ± 30.61 ng/ul, 25.24 ± 11.84 ng/ul and 25.37 ± 10.54 ng/ul, respectively. UV absorbance mean was different from automated microfluidic electrophoresis (p<0.0001) and qPCR (p<0.0001). No difference was observed between automated microfluidic electrophoresis and qPCR (p=0.96). Correlations coefficient (r) were: 0.65 (p=0.0002) for automated microfluidic electrophoresis and UV absorbance, 0.67 (p=0.0008) for UV absorbance and qPCR, and 0.92 (p=1.35x10-12) for automated microfluidic electrophoresis and qPCR. Bias (%) ± sd and 95% limits of agreement were 53.98% ± 49.57 and -43.18% to 151.1% between automated microfluidic electrophoresis and UV absorbance, 52.47% ± 51.06 and -47.6% to 152.6% between UV absorbance and qPCR, and -2.01% ± 17.25 and -35.86% to 31.76% between automated microfluidic electrophoresis and qPCR, respectively. **Conclusion:** Here we observed a higher agreement between automated microfluidic electrophoresis and qPCR for genomic DNA quantification. Conversely, both methods showed different results from UV absorbance method. Probably, DNA quantification by UV absorbance is being overestimated due to the co-extraction of RNA. Automated microfluidic electrophoresis and qPCR seems to be comparable (and reliable) quantification methods. However, both methods have their particularities. Automated microfluidic electrophoresis would be adequate for high molecular weight DNA and when information about the DNA integrity is needed (e.g., target NGS sequencing). On the other hand, qPCR would be adequate for fragmented DNA and when information about the presence of PCR inhibitors co-purified during the extraction is necessary (e.g., liquid biopsies and FFPE tissue).

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Evaluation of PD-L1 Expression by Immunohistochemistry with Fluorescent Antibodies from Flow Cytometry

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Background: We describe the use of peroxidase and alkaline phosphatase conjugates of anti-FITC, -PE, -APC and -PerCP antibodies in order to enable the use and reuse of primary antibodies from flow cytometry in immunohistochemistry with cryosections. While multi-parameter flow cytometry is very effective at evaluating the expression of proteins on particular cell types, it does not provide any information about the relative locations of cells in tissues. This can be overcome by fluorescence microscopy, many of the common fluorophores used in flow cytometry are bright but prone to rapid photobleaching, and autofluorescence can also be a problem. Immunohistochemistry with conventional secondary anti-mouse, -rat, etc. antibodies can overcome issues with autofluorescence, but many primary antibodies are from the same species and of the same isotype, which makes it challenging to combine two in a dual color stain. **Methods:** We used anti-fluorescent tag antibodies in order to reuse antibodies from flow cytometry and evaluated the expression of PD-L1 and other markers in tissues by dual color immunohistochemistry. Briefly, cryosections were fixed and endogenous peroxidase was inactivated with sodium azide followed by hydrogen peroxide. Sections were blocked and then stained with the primary antibodies followed by the anti-fluorescent tag antibodies. The substrate for alkaline phosphatase (HistoMark RED) was applied first to generate a magenta signal followed by a substrate for peroxidase (TrueBlue or DAB) to generate a blue or brown/black signal. Afterwards, the molecule proflavine could be used to stain the nuclei yellow. **Results:** On human tonsil sections, it was possible to visualize the expression of PD-L1 in relation to T cell subsets, B cells and other cell types. Weaker expression was simulated by using reduced amounts of primary antibodies such that it was no longer possible to distinguish the staining from autofluorescence by fluorescence microscopy. In those cases, the combination of anti-fluorescent tag antibodies and the TrueBlue substrate was generally still capable of detecting expression. **Conclusion:** Anti-fluorescent tag antibodies enable the use and reuse of antibodies from flow cytometry in immunohistochemistry in order to relatively quickly evaluate where expression is taking place without having to use a separate set of antibodies.

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Analytical Validation of a MALDI-ToF Pharmacogenomic Assay

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2018 AACC Abstract
RELEVENCE: Genotyping of pharmacodynamically relevant SNPs is routinely performed when optimizing patient care for therapeutic drugs and has also been hypothesized to be important to predict susceptibility to drug-misuse disorders. Recent combinations of pharmacogenomic biochemical tools and mass spectrometric methods have allowed for sensitive, economical, and rapid processing of patient samples for pharmacogenomic patient profiling in the clinic. Mass Spectrometry is an analytical technique exceptional for a high level of sensitivity which enables successful analysis of sample sizes up to 2 orders of magnitude smaller than that of traditional RT-PCR. Here, we compare the Agena MassARRAY MALDI-ToF analyzer using the PGx74 SNP panel for genotyping of patient samples previously characterized by RT-PCR. **OBJECTIVE:** The goal of this study was to validate the analytic accuracy and reproducibility of the Agena MassARRAY instrument and PGx74 assay via comparison to previously tested DNA samples with known genotypes. The ultimate goal is to develop a prospective tool to guide opioid usage in patients seeking care from Pain Management providers. **MATERIALS AND METHODS**: We assessed the ability of the Agena MassARRAY 4.0 MALDI-ToF MS instrument to detect SNPs known to be associated with opioid metabolism and response. The Agena PGx74 panel detects wild type and variant SNP sequences as well as copy number variants in 20 genes, including *ABCB1*, *APOE*, *COMT*, *CYP1A2*, *CYP2B6*, *CYP2C19*, *CYP2C9*, *CYP2D6*, *CYP3A4*, *CYP3A5*, *DRD2*, *F2*, *F5*, *GLP1R*, *MTHFR*, *OPRM1*, *PNPLA5*, *SLCO1B1*, *SULT4A1*, and *VKORC1*. Archived DNA samples which were previously tested for pharmacogenomic-related SNPs using TaqMan assays were used to assess performance of the Agena PGx74 assay. After multiplex PCR of target regions, a single base extension reaction was performed that allows for simultaneous SNP and copy number variant detection. Accuracy of the PGx74 assay was determined as percent concordance with previous genotypes. **RESULTS**: Ten samples with known *CYP2C19* genotypes were analyzed using the MassARRAY PGx74 multiplex panel. Three specimens were analyzed four times. Two of the three generated *CYP2C19* results that matched the known genotype on all four replicates. The third specimen generated "no call" results on two of the four replicates. The remaining seven specimens were analyzed twice. Six of these generated concordant *CYP2C19* results on both runs while the remaining specimen generated "no call" results on both runs. **CONCLUSIONS:** The Agena MassARRAY demonstrated accuracy and reproducibility in high quality, previously characterized DNA samples. In lower quality samples, the MassARRAY generated "no call" results rather than incorrect results. The combination of highly sensitive mass spectrometric methods and high-throughput pharmacogenomic biochemical tools may allow for the estimation of patient risk for adverse outcomes following use of opioid therapeutics. The ability to rapidly genotype these specimens is of great utility to providers and patients seeking safe and effective pain relief.

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Development of a Type I Diabetes Genetic Risk Array

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Background: Differentiating between Type 1 diabetes (T1D) and Type 2 diabetes (T2D) is challenging due to the increasing incidence of childhood obesity blurring the traditional T1D versus T2D timelines. More young people are getting T2D and T1D can occur at any stage in life, but treatment and patient care pathways are very different depending on diabetes type. Besides this, there has been an increasing number of cases of T1D occurring at old age. Currently available diagnostic tests have several limitations in accurately diagnosing diabetes subtypes. Up to 15% of young adults are wrongly classified and treated. The aim of this study was to consider genetic predisposition as an aid to improve diabetes classification. Genetic predisposition to diabetes is largely determined by the presence of human leukocyte antigen (HLA) genes. A number of Single Nucleotide Polymorphisms (SNPs) that tag these genes have been shown to identify increased risk. Genome-wide association studies have identified additional non-HLA SNPs, robustly linked with T1D. Combining these, a 10 SNP genetic risk score (GRS) was developed which can aid discrimination between T1D and T2D, particularly when used in conjunction with clinical features and autoimmune markers. **Methods:** The assay employs multiplex Polymerase Chain Reaction (PCR) coupled to Biochip Array Technology (BAT, Randox Laboratories Ltd, Crumlin, UK) to genotype 10 SNPs associated with T1D (Oram *et al*.,2016). Multiplex SNP-specific PCR amplicons are hybridised and spatially separated onto a grid of discrete test regions on a biochip, followed by chemiluminescent detection on the Evidence Investigator analyser. Assay run time is <3 hours from DNA template to generation of SNP genotypes. Assay optimisation and specificity was realised using pre-characterised DNA samples and initially validated by testing DNA samples (n=259) provided by University of Exeter, with results compared against genotypes using an alternative method. **Results:** The Type 1 Diabetes GRS array is capable of rapidly detecting all 10 SNPs associated with T1D. Using the biochip array, all 259 samples (2590 genotypes) were in agreement (99.9%) with genotypes predicted by University of Exeter. Samples tested covered all the genotypes linked to 10 SNPs. **Conclusion:** The Type 1 Diabetes GRS array provides a rapid and reliable genotyping test for detecting 10 SNPs associated with T1D. Through an associated algorithm, the array can generate a T1D Genetic Risk Score, which in conjunction with conventional methods, can distinguish T1D from other subtypes. This assay has potential to prevent misdiagnosis of diabetes and facilitate improved diabetic patient management.

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Alterations in hepatic oxylipins, oxidized metabolites of n3 and n6 PUFAs, in liver injury caused by alcohol consumption

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Background: Alcoholic liver disease (ALD), a significant health problem, progresses through the course of several pathologies including steatosis, steatohepatitis, fibrosis, and cirrhosis. There are no effective FDA-approved medications to prevent or treat any stages of ALD, and the mechanisms involved in ALD pathogenesis are not well understood. Bioactive lipid metabolites play a crucial role in numerous pathological conditions, including liver diseases, as well as in the induction and resolution of inflammation. **Aim/Objective**: Herein, a hepatic lipidomic analysis was performed on a mouse model of ALD with the objective of identifying novel metabolic pathways and lipid mediators associated with alcoholic steatohepatitis, which might be potential novel biomarkers and therapeutic targets for the disease. **Methods:** C57BL6/J male mice were fed *ad libitum* control or ethanol (EtOH) liquid diets that contained a high percentage of unsaturated fat (USF, corn-oil enriched) or saturated fat (SF, medium chain triglyceride oil-enriched) for 10 days, followed by a single EtOH administration by oral gavage. Liver steatosis, inflammation, and injury were evaluated. Hepatic lipidomic was performed by HPLC analysis. Mass spectra for each detected lipid mediator were recorded to verify the identity of the detected peak in addition to MRM transition and retention time match with the standard. The data were collected and the MRM transition chromatograms were quantitated. The internal standard signals in each chromatogram were used for normalization for recovery as well as for relative quantitation of each analyte. Statistical significance was determined using Two-Way Analysis of Variance (ANOVA). A p-value of < 0.05 was considered statistically significant. **Results:** We found that EtOH and dietary USF, but not SF, resulted in more severe liver damage, including elevated plasma ALT levels (a marker of liver injury), enhanced hepatic steatosis, oxidative stress, and inflammation. qRT-PCR analysis for macrophage type M1 and M2 cytokine gene expression revealed that M1-associated proinflammatory cytokines (Tnf-α and Il-1beta) were elevated in mice fed the USF+EtOH diet but showed no changes in M2-associated (Tgf-beta and Arg-1) cytokine gene expression. We then performed a targeted lipidomic analysis to measure hepatic levels of bioactive lipid metabolites generated from ω3 and ω6 PUFAs, predominantly via three major enzymatic pathways, LOX, COX and CYP/sEH. A total of 17 oxylipins out of 79 detected metabolites were found to be significantly different (increased) in the USF+EtOH compared to SF+EtOH group, and 21 lipid metabolites were different (19 increased and 2 decreased) between the USF+EtOH and control USF-diet fed animals. Liver injury induced by USF+EtOH was associated with increased levels of oxylipins generally involved in pro-inflammatory responses, including 13-hydroxy-octadecadienoic acid, 9,10- and 12,13-dihydroxy-octadecenoic acids, 5-, 8-, 9-, 11-, 15-hydroxy-eicosatetraenoic acids, and 8,9- and 11,12-dihydroxy-eicosatrienoic acids, in parallel with an increase in pro-resolving mediators, such as lipoxin A4, 18-hydroxy-eicosapentaenoic acid, and 10S,17S-dihydroxy-docosahexaenoic acid. **Conclusion:** The identification of pro- and anti-inflammatory and pro-resolving eicosanoids/oxylipins associated with EtOH-induced liver damage may shed new light into the molecular mechanisms underlying ALD development/progression and might be novel biomarkers and potential new therapeutic targets for ALD. Therefore, further mechanistic studies are warranted.

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Comparison of the *Realtime* HPV HR-S Detection with the Cobas 4800 HPV test for the detection of high-risk types of human papillomavirus

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Background: The *Realtime* HPV HR-S Detection (SEJONG BIOMED, Paju, Korea) is one of the recently developed assays, which is a real-time PCR based test designed for detecting 14 types of high-risk (HR) HPVs (type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). This study was to compare the performance of *Realtim*e HPV HR-S Detection to Cobas 4800 HPV test (Roche Molecular Systems, Branchburg, NJ, USA) for the detection of high risk (HR) HPV. **Methods:** A total of 334 cervical swab specimens were retrospectively collected from patients whose mean age was 43.3 ± 12.0 years (22-90 years) between June and September 2016. We tested all the specimens by *Realtime* HPV HR-S Detection and Cobas 4800 HPV test. HPV DNA sequencing was subsequently analyzed to confirm the discordant results. HPV distribution by age, results of Pap test and cervical biopsy for 303 patients was also analyzed and the sensitivity and specificity for high risk lesion and squamous cell carcinoma (SCC) were analyzed. **Results:** Cobas 4800 HPV test one of 13 HR HPV types in 62.0% of specimens, while Realtime HPV HR-S Detection detected in 62.6% of specimens. The overall agreement rate between the assays was 95.2% with 0.937 kappa coefficient. One of the discordant samples was revealed that the result from Cobas was equal to sequencing and the rest 15 samples were revealed that the results from Realtime HPV were equal to sequencing. Sensitivity and specificity of 16, 18 and other high HPV detections were high enough (Cobas 4800: 91.7-100.0% and 98.0-100.0%, and Realtime HPV HR-S Detection: 98.6-100.0% and 100.0%). Sensitivity and specificity for high grade lesion and SCC were similar between at the two tests; 76.9% and 41.9% by Realtime HPV HR-S Detection vs. 80.8% and 43.0% by Cobas 4800 HPV test.For the clinical performance, we calculated sensitivity and specificity of each two tests for pathologic finding of high grade lesion and SCC. *Realtime* HPV HR-S Detection showed 76.9% (56.4-91%) of sensitivity and 41.9% (36.0-47.9%) of specificity while Cobas 4800 HPV test showed 80.8% (60.6-93.4%) and 43% (37.1-49.0%) of those. **Conclusion:** HPV test is getting important at the risk evaluation of cervical cancer. With a methodological development of HPV DNA detection, HPV HR typing is required as well as an accurate HPV detection. In addition, the test has to be validated in a clinical aspect with sensitivity and specificity for the clinical use. *Realtim*e HPV HR-S Detection showed a high agreement rate with Cobas 4800 HPV test and a similar analytical effectiveness with high sensitivity and specificity. Considering the results, *Realtim*e HPV HR-S Detection could be a reliable laboratory testing method for the screening of HPV infections

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An evaluation of Clostridium Difficile Polymerase Chain Reaction on the Roche Cobas 4800.

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Introduction

We compared our current method of toxigenic Clostridium Difficile (*C diff)* testing against the Roche Cobas 4800 system and CE marked Roche C diff test. Our current methodology, validated for in-house use is the TIB MOLBIOL (Berlin, Germany) LightMix *C. difficile* Test, optimised for the Roche LightCycler 2.0 (Basel, Switzerland). Sample preparation of the stool is performed on the Qiagen EZ1 (Venlo, Netherlands). In contrast, the Cobas 4800 system comprises the extraction and purification x480 unit while amplification, detection and result rendition takes place on the z480. The only human intervention cl-required is to transfer the prepared PCR plate from the x480 to the z480 unit.

Materials and methods

We tested 83 anonymised stool samples either retrospectively or as split samples on the Cobas 4800, in accordance with the manufacturer's instructions. The study also included samples spiked with ATCC strains for Ribotype 027, 405 and AI-56. A lower limit of detection challenge was performed using a serially diluted patient sample, ATCC spiked negative stool and ATCC Ribotype 027 spiked negative stool. A challenge for cross-reactivity was performed by spiking samples with up to 1 McFarland equivalent concentrations of various organisms. Assay precision was evaluated using the cross-threshold values of the negative control, internal control values and the positive control values.

Result

The analytical sensitivity and specificity for the Roche Cobas *C diff* test was 96.4% and 96.1% respectively. The lower limit of detection was determined to be less than 100 CFU/mL which is within the manufacturer's claim. Inter-assay precision yielded a coefficient of variation of 1.34% and 1.53% for the internal and positive controls respectively. No false positives were detected with negative samples spiked with *E. coli, B fragilis* and *P mirabilis*.

Conclusion

The Roche Cobas 4800 *C diff* test is able to provide equivalent diagnostic performance on a platform that requires minimal human intervention. Compared to the current method which encompasses several touch-points which increases risk for contamination and mistakes to occur, the 4800 system allows the operator to focus on more complex activities while maintaining high service levels and customer expectations.

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Unbalanced inherited complex chromosome rearrangement in a child with congenital malformations and developmental delay

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Background: Complex chromosomal rearrangements are rare structural aberrations that involve three or more chromosomes and greater than two breakpoints. They are defined as balanced if the chromosomes have the normal chromosomal complement, or unbalanced if there is additional or missing material. Some carriers of complex chromosomal rearrangements appear to have apparently balanced rearrangements but presents abnormal phenotypes. Abnormal phenotypes are associated to cryptic deletions or duplications, or disruption of genes at the site of recombination, which cannot detected by karyotype analysis. With the introduction of microarray analysis as the diagnostic test, large numbers of submicroscopic, pathogenic copy number variants (CNVs) have been uncovered. **Objective:** To report a case of unbalanced complex chromosomal rearrangement paternally inherited detected in a child with multiple malformations. **Case report:** We report 1-year-old boy with psychomotor development cardiopathy, seizures and syndromic facies. He was born to nonconsanguineous and asymptomatic parents, with no history of obortions. Cytogenetic analysis with G-banding was performed on peripheral blood from the child. An apparently balanced complex rearrangement was found: 46,XY,ins(3;7)(p25;q11.23q21.2)ins(1;3)(q32;p24.1p25). Parental chromosomes analysis were performed. The mother's karyotype was normal. The father was found to have a similar apparently balanced complex karyotype: 46,XY,ins(3;7)(p25;q21.11q21.3)?ins(1;3)(q32;p24.2p25). The proband's array-based comparative genomic hybridization (array-CGH) analysis revealed two pathogenic alterations: a 17.4 Mb duplication in 3p26.3-p24.3 [arr[hg19] 3p26.3p24.3(611,636-18,039,381)x3 and a 16.6 Mb deletion in 7q21.11q21.3 [arr[hg19] 7q21.11q21.3 (78,899,856-95,550,252)x1]. Parental array-CGH analysis were normal. The proband's analysis is the first case in which occurred simultaneously a 3p26.3-p24.3 duplication and a 7q21.11-q21.3 deletion, therefore, we cannot associate its findings to any case already described. However, many features described in the literature for the alterations separately are coincident and correlated with the clinic of the evaluated patient. The complex rearrangements identified by karyotype analysis were interchromosomal insertions involving chromosomes 1, 3 and 7. A balanced, interchromosomal insertion is characterized by one chromosome with an interstitial deletion and another chromosome with na interstitial insertion. As there is no reciprocal segment involved, the imbalances that result from segregation can be a segmental monosomy (loss) or trisomy (gain). There is no question that the array-CGH in the clinic has revolutionized the way segmental aneusomies are detected. Developments in array platforms and bioinformatic tools have improved our ability to detect CNVs even in apparently balanced structural rearrangements. However, in spite of these amazing new technologies, a major roadblock remains unchanged. When a copy number gain is detected, it is impossible to determine where in the genome the additional material resides using the array data alone. This case highlights the importance of an integrated approach to cytogenetic analysis and array-CGH analysis.

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HLA-tagging SNP may not be suitable for Celiac Disease risk prediction in Brazilian population

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Background: Celiac disease (CD) is a gluten-sensitive autoimmune disorder, estimated to affect 1:100 people around the world. In Brazil, according to the National Federation of Celiac Associations of Brazil (FENACELBRA), the historical miscegenation may be responsible for a similar proportion of CD cases, although there are no epidemiological studies available to confirm this estimative. CD is the result of the combination of genetic (human leukocyte antigen: HLA) and environmental (gluten) factors. HLA class II genes are strongly associated with CD predisposition, especially the DQ2.5 (DQA1*05/DQB1*02), DQ8 (DQA1*03/DQB1*0302) and DQ2.2 (DQA1*02/DQB1*02) haplotypes. Almost 95% of CD patients carry at least one of the two risk molecules (DQA1 or DQB1). However, up to 40% of healthy people also carries one of these risk factors and their presence alone is not diagnostic of CD. Testing for HLA risk molecules is routinely performed by different methods, e.g. Tagging SNP, PCR-SSOP and MLPA. In our lab, an in-house assay and Tagging SNP are commonly used. The usage of six HLA-tagging SNPs (Monsuur *et al,* 2008) has proven high specificity and sensitivity (>95%) in European celiac populations, but there are no studies using Brazilian population or comparing these methods. **Objective:** This report compares Tagging SNP and our in-house assay in order to assess the agreement between both methodologies as predictive tools for CD risk. **Methodology:** Genomic DNA was extracted from 13 blood or buccal swab samples using QIAGEN kits. The HLA-tagging SNP was performed as Monsuur *et al*. (2008). The in-house assay consists of DQ2 (DQA1*0501 and DQB1*0201) and DQ8 (DQB1*0302) detection, through allele-specific PCR and fragment analysis followed by AmbiSolv® (Thermo Fisher), respectively. **Results and Discussion:** We obtained seven discordant results (54%) between both methodologies. Two patients presented high risk for CD when submitted to our in-house assay but low risk by Tagging SNP. One of these patients was also analyzed by PCR-SSOP and presented high risk for CD. Five patients presented low risk for CD when submitted to the in-house assay but high risk by Tagging SNP. **Conclusions:** The demand for CD genetic tests has increased in the last years, and reliable determination CD genetic predisposition can avoid unnecessary biopsy and gluten-free diet prescriptions. HLA-tagging SNP is a cost-effective population screening method for CD considered as highly accurate, but it has been only validated in Finnish, Hungarian and Italian populations. Our results are a strong indicative that these six HLA-tagging-SNPs may not be suitable for Brazilian population analysis. More patients will be analyzed and also tested by PCR-SSOP to confirm the CD risk correlations between methodologies.

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Marfan syndrome screening using NGS and MLPA in Brazilian patients

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Background: Marfan syndrome (MFS; MIM# 154700) is a rare, autosomal-dominant connective tissue disorder that is caused in the majority of cases by mutations in fibrillin 1 (*FBN*1; MIM# 134797). This gene comprises 66 exons located in the long arm of chromosome 15 at 15q15-q21.1. Mutations in the transforming growth factor-β receptor 1 and 2 genes (*TGFBR*1 and *TGFBR*2) on chromosome 3 have also been identified in a subset of MFS patients who did not carry *FBN*1 mutations. The disorder is characterized by variable manifestations in the cardiovascular, ocular and skeletal systems. Diagnosis is based on familial history of MFS, *FBN*1 mutation analysis and specific phenotypic characteristics, including ectopia lentis, aortic dilatation or dissection and skeletal features. The incidence is currently estimated to be of 1-3 per 10000 individuals, although this could be under-estimated due to misdiagnosis. Next Generation Sequencing (NGS) and Multiplex Ligation-Dependent Probe Amplification (MLPA) have been introduced into DNA diagnostic laboratories for the detection of molecular genetic alterations that are of diagnostic and prognostic significance, such as SNPs and large gene duplications and deletions. **Objective:** Investigate the presence of gene mutations in eight Brazilian patients with familial history and clinic diagnostic of MFS. **Methodology:** Patients were tested using the commercial MLPA kits P065-B1 and P066-B2 (MRC-Holland) for *FBN*1, *TGFbR*1 and *TGFbR*2, following manufacturer's instructions and the *FBN*1 gene was sequenced on Ion Torrent PGM sequencer. Variant analysis was performed using Ion Reporter software and detected variants were classified according to 2015 ACMG-CAP guideline. Furthermore, linkage analyses were performed with genetic markers near *FBN*1 allele related to the altered phenotype. **Results and Discussion:** One patient presented a large disruptive heterozygous deletion in *FBN*1 gene from exons 50 to 66. No alterations were detected by MLPA for *TGFBR*1 and *TGFBR*2. A total of 14 (13 SNPs and one deletion) variants were detected considering all patients with four novel variants not currently annotated in public databases such as ClinVar and dbSNP. Three patients presented novel likely pathogenic variants in exons 14, 29 and 64. One patient presented a novel pathogenic variant in exon 16. Eight variants were benign or likely benign and the remaining 2 variants were of unknown significance. No alterations were found for three patients; therefore, the genetic factor contributing for the disease is probably located in their intronic or regulatory regions or in genes other than those analyzed. **Conclusions:** MFS is a genetic disorder with considerable morbidity and mortality, which early diagnosis is essential for the prevention of aortic events. The description of novel variants related to MFS is an important support for medical counseling and diagnosis.

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Analyses on the genotype and phenotype of pediatric deaf patients with cochlear implantation

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Background: National surveys show that the number of handicapped people with hearing impairment in China has reached to more than 27 million. Cochlear device implantation (CDI) is still the only reliable choice for hearing treatment in severe sensorineural hearing loss. This study sought to screen the frequency of mutations and the main types of the mutations, to provide theoretical basis for further analysis on the differences in hearing and speech rehabilitation after CDI in children with different genetic mutations and to provide clinical guidance for children with deafness who need CDI. **Methods:** A total of 42 patients that did not pass newborn hearing screening and were diagnosed as congenital infant deaf had peripheral blood samples collected at the First Affiliated Hospital of Anhui Medical University. Genetic testing was performed by fluorescence PCR on three high-frequency deafness susceptibility hot spot gene mutations: GJB2, SLC26A4 (PDS) and mitochondrial mtDNA 12s rRNA. **Results:** Four out of 42 patients (9.52%) had GJB2 235delC heterozygous mutation, 8 (19.05%) had SLC26A4 mutation (1 case of IVS7-2 A> G homozygous mutation, 1 case of 1174A> T homozygous mutation, 1 case 2168 A> G homozygous mutation with IVS7-2 A> G heterozygous mutation, 3 cases of IVS7-2 A> G heterozygous mutation, and 2 cases of 1174 A> T heterozygous mutation) and 2 (4.76%) had mtDNA 12s rRNA 1555A> G heterozygous mutation. **Conclusion:** Among the 42 cases of pediatric patients with congenital deafness and went through cochlear implantation, the detection rate of the three common deafness gene mutations in China was 33.33%, of which the mutation of SLC26A4 had the highest frequency, GJB2 gene was the next, and the mutation of mitochondrial gene was not common. This study provided a theoretical basis for further analysis of children with different gene mutations in hearing and speech rehabilitation after cochlear implantation, and provides clinical guidance for children with deafness who need cochlear implantation.

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limit of detection of the molecular assays mix® modular giardia for the study of giardia lamblia in coprological samples

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Blackground: The diagnosis of persistent diarrhea due to *Giardia lamblia* can be complicated by the low parasitic burden. The most used diagnostic technique by laboratories, is microscopy, due to its low cost and simplicity. However, traditional microscopy requires time and experience,In recent years, real-time PCR has become an important alternative for the diagnosis of intestinal parasitosis, especially due to its greater specificity and sensitivity to microscopy, an aspect that has been evaluated in patients with acute diarrhea.The aim is to evaluate the detection limit of the LightMix® Modular Giardia Roche Diagnostics molecular technique for the detection of *Giardia lamblia* cysts.**Methods:** We studied 3 samples of stools parasitized with *Giardia Lamblia* and considered microscopically as high parasitic concentration (~840cysts/µL, 1), medium (~490cysts/µL, 2) and very low (~30cysts/µL,3). Serial dilution was performed in duplicate in each sample, microscopically quantifying each one (number of *Giardia lamblia* cysts per microliter) in Burker's chamber. The limit of detection was defined as the lowest dilution that showed a positive result in 100% of the cases.The extraction phase was carried out in MagnaPure equipment (Roche Diagnostics) and for this the samples were diluted 1/10 in STAR Buffer and subsequently pre-treated with MagNAPure Bacteria Lysis-Buffer.Molecular amplification was performed by real-time PCR (Roche Diagnostics) amplifying a 62 bp fragment of the 18s rRNA gene from *Giardia* on a Cobas z480 device.**Results:**

Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
	C-->Dc	C-- >Dc	C-- >Dc	C-- >Dc	C-->Dc	C-->Dc	C-- >Dc	C-->Dc	C-- >Dc
Sample, 1	843-- >31.63	428-- >33.83	227-- >34.32	109-- >35.30	52-- >35.67	23-- >36.55	11-- >37.21	5-->-	3-->-
848-- >31.61	431-- >33.85	236-- >34.34	113-- >35.32	55-- >35.71	26-- >36.68	12-- >37.23	5-->-	1-->-	
Sample, 2	490 -->32.60	241-- >34.10	123-- >34.98	61-- >35.23	30-- >36.13	13-- >37.17	5-->-	2-->-	
493-- >32.61	244-- >34.07	125-- >35.07	63-- >35.25	32-- >36.23	12-- >37.21	6-->-	4-->-	1-->-	
Sample, 3	31-- >36.14	13-- >37.19	8-->-	5-->-					
33-- >36.25	14-- >37.06	7-->-	4-->-	1-->-					

C: Concentration (cysts/µL)Dc: Detection cycleThe median was 12 (11.7-13.3).**Conclusion:** The LightMix® Modular Giardia has a detection limit between 12-13cysts/µL. Therefore, samples parasitized with a low number of cysts of *Giardia lamblia* could be negative in the molecular study of the stools samples of these patients. In addition, the small volume of sample used in the extraction phase, together with the difficulty of breaking the cysts in the pretreatment, may limit the usefulness of molecular techniques as an alternative to conventional microscopy.

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Proficiency testing in NSCLC-related multigene molecular detection by next generation sequencing in China

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Background: Non-small cell lung cancer (NSCLC) is heterogeneous group of carcinomas and stands 70%-80% of cases in lung cancer. As targeted therapies based multiple-gene testing have improved the outcomes of NSCLC patients and decreased cost of next-generation sequencing (NGS) technology have been widespread used as clinical diagnostic assays in numerous laboratories, the accuracy of oncogenic driver mutation detection becomes an indispensable component in genome-directed cancer therapy. Hence, National Center for Clinical Laboratories (NCCL) organized a proficiency testing (PT) for NGS-based multiple genetic detection in NSCLC in 2017,

on the purpose of evaluating the multiple gene testing capability in China. **Methods:** Mimicking NSCLC-related clinical samples were provided to 101 participating laboratories, each with a mock clinical case report. Laboratories were required to use their routine NGS methods to perform the genetic testing and submitted all the variants involved in their detection range within three weeks. Results were evaluated based on predefined marking criteria, the qualified score was set at higher than 90 points. **Results:** Overall, ninety-five laboratories reported their results on schedule. Fifty-two laboratories were considered as qualified while forty-three had completely correct results. More than three hundred errors were divided into 4 parts: 32 false-negatives, 224 false-positives,48 mutation errors and 54 nomenclature errors. The detection rates of SNVs and gene fusion were more than 85% while laboratories had a poor ability in detecting indels, the detection rate of *ERBB2* c.2326delGinsTTAT (p. Gly776delinsLeuCys) was only 62%. **Conclusion:** Our PT results indicated that the detection capability of Chinese laboratories must be improved to further increase the accuracy of NGS-based multigene molecular analysis to ensure reliable results for selection of precision therapy.

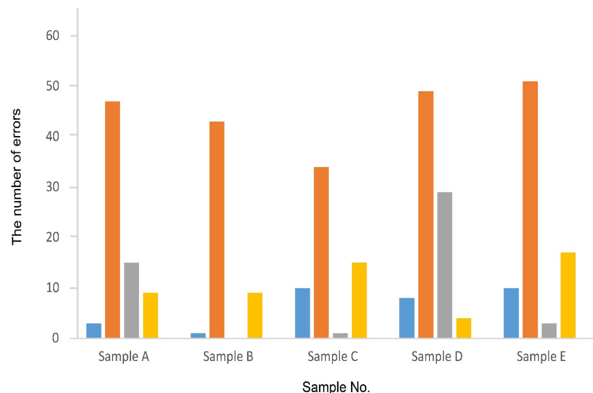

Figure 1: The distribution of errors for each sample in the PT scheme. Four kinds of errors include false-negatives(blue), false-positives(orange), mutation errors(grey)and nomenclature errors(yellow).

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Quantitative detection of *JAK2*-V617F mutation using droplet digital PCR for molecular diagnostics of myeloproliferative neoplasms

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Background: Myeloproliferative neoplasms (MPNs) are chronic neoplastic disorders defined as abnormal increases in mature peripheral blood cells, resulting in the aberrant clonal proliferation of hematopoietic progenitors. MPNs consist of chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocytosis (ET), and myelofibrosis (MF). Karyotyping and BCR-ABL rearrangements tests are indicated for diagnostic and prognostic of these disorders. Under negative results, investigation of V617F mutation in the *JAK2* gene is required. JAK2 is a tyrosine kinase that act in signal transduction in hematopoietic cells. The V617F mutation results in the production of a constitutively activated JAK2 protein, which seems to improve the cell survival and increase production of blood cells. This mutation is found in approximately 96% of patients with PV, 50% of individuals with ET and primary MF. Besides the qualitative detection of a V617F mutation, quantification of mutated allele burden provides useful information for classifying subgroups of MPNs and to predict disease-associated outcomes. **Objective:** The aim of this study it was to validate the quantitative detection of the V617F mutation through a high-sensitivity methodology (droplet digital PCR-ddPCR) improving the molecular diagnostics of myeloproliferative neoplasms. **Methods:** Wild-type (≅4000 copies/reaction) and mutated alleles (≅5000 to 0 copies/reaction) of synthetic DNA were combined to stablish the limit of quantification (LOQ) of the test. Blood samples were obtained from health donors (n=2) to determine the false-positive rate and limit of detection (LOD). Twenty-six MPNs samples were selected based on previous results of qualitative V617F test (qPCR) to demonstrate the clinical performance of the ddPCR. DNA samples were collected using QIAamp DNA Mini Blood or Puregene Blood Kits (QIAGEN) and yield was measured by Nanodrop (ThermoFisher). DNA samples (10ng/uL) were submitted to ddPCR using V617F JAK2 Digital PCR Mutation Detection Assay (ThermoFisher) and runned at QX100 Droplet Digital PCR System (BioRad). **Results:** ddPCR detected the presence of the V617F mutation in all dilutions down to 0.06% of mutant alleles. However, LOQ of V617F was achieved at 0.1%, which approximates to 3.4 copies/reaction of mutant alleles. The false-positive rate it was 0.05% and LOD stablished was ≥3 positive droplets to call a V617F positive result (95% CI). To further assess specificity of ddPCR, JAK2 V617F allele burden was measured in 26 DNA samples previously found positive (n=13) and negative (n=13) by qPCR. All positive samples could be classified as positive by ddPCR as well showing different frequencies of allele burden (1.4% to 93.1% of mutant alleles). Among negative samples, 1/13 (7%) patient was positive by ddPCR (0.26% of mutant alleles). This patient had a previously negative BCR-ABL test. **Conclusions:** Our data showed that ddPCR cutoff value was 0.1% of mutant allele in a background of wild-type alleles for an accurate quantitation of JAK2 V617F. In addition, this methodology showed high sensitivity than qPCR for at least 7% of cases. ddPCR was able to identify low levels of V617F mutation from one patient who was negative for qPCR. Therefore, quantitative detection of V617F can be used for prognosis and minimal residual disease monitoring in MPNs patients.

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Regulatory role and clinical significance of circular RNAs in Spinocerebellar ataxia type 3

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Backgrounds: Spinocerebellar ataxia type 3/Machado-Joseph disease (SCA3/MJD) is the most common autosomal dominant spinocerebellar ataxia and one of many inherited polyglutamine (polyQ) neurodegenerative diseases. Nevertheless, the exact mechanism of the disease still remains ambiguous. At present, circular RNAs (circRNAs) have been attracting extensive research interest in different human diseases, which emerged as new key regulators via different biological functions in genetic and epigenetic processes, but it remains largely unknown if they are correlated with SCA3/MJD pathogenesis. Therefore, the objective of this work was to investigate the significance and potential role of circRNAs in SCA3/MJD. **Methods:** Here, we adopted next-generation sequencing(NGS)to examine the expression profile of circRNAs and mRNAs in cerebrospinal fluid(CSF)samples and peripheral blood samples from 11 SCA3/MJD patients and 10 healthy controls. Next, quantitative real-time reverse transcription polymerase chain reaction(qRT-PCR) was performed to validate NGS data. ROC analysis was also used to evaluate the predictive power of candidate circRNAs. In order to elucidate potential functions and signaling pathways involved in the pathogenesis of SCA3/MJD, we applied Gene ontology(GO) and Kyoto Encyclopedia of Genes and Genomes(KEGG) pathway analysis of the differentially expressed mRNAs and parental genes of circRNAs. Furthermore,after the knockdown or overexpression of candidate circRNA, MTT assay along with flow cytometric assays were used to assess changes in cell viability as well as apoptosis , and Western blot was performed to analyze the disease protein—polyQ-ataxin3 expression in SY-SH5Y/SCA3 cell models. **Results:** Our results showed that circRNAs and mRNAs profiles presented a total of 262 circRNAs and 1001 mRNAs commonly expressed in both CSF samples and peripheral blood samples of SCA/MJD patients. Among them, 14 circRNAs as well as 429 mRNAs were upregulated and 42 circRNAs as well as 549 mRNAs were downregulated in SCA3/MJD group. The expression level changes of 5 differentially expressed circRNAs estimated by qRT-PCR were in accord with NGS data. Moreover, hsa_circ_0019149 (AUC:0.953; 95% CI: 0.911–1.005) were the most upregulated and significantly associated with SCA3/MJD, which could be identified as novel candidate diagnostic biomarker for the disease. Significantly enriched signaling pathways were involved in apoptosis, protein degradation, etc. The viability of cells increased markedly and polyQ-ataxin3 expression decreased following the knockdown of hsa_circ_0019149, whereas overexpression of hsa_circ_0019149 had the opposite effects on cells. **Conclusion:** These findings were the first report of differentially expressed circRNAs in SCA3/MJD, indicating a possible role for circRNAs as potential dynamic monitoring progress biomarkers and possibly original diagnostic or therapeutic targets of the disease. Also, our results provided novel insights into the mechanisms of the pathological process as well as important cues for further functional studies of the disease.

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Ectopia Lentis diagnosed by NGS sequencing of a new pathogenic variant of the FBN1 gene.

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Background: Marfan syndrome (MS) is a systemic connective tissue disease characterized by a variable combination of cardiovascular, musculoskeletal, ophthalmological and pulmonary manifestations. In most cases it is due to mutations in the FBN1 gene (15q21), which codes for an essential connective tissue protein, fibrillin-1. There are identified borderline forms of the disease secondary to mutations in the TGFBR2 gene (3p22), which codes for TGF-beta´s receptor. On the other hand, Isolated *Ectopia Lentis* (IEL) is a rare eye disorder characterized by subluxation of the lens and a significant visual acuity decrease. Its prevalence is unknown, having been described around 90 cases to date, mainly in Europeans. Other manifestations include: congenital anomalies of the iris, spherophakia, abnormal iridocorneal angle, iridodonesis, coloboma of the lens, refractive errors, cataracts of early onset, amblyopia, displacement of the pupils (*ectopia lentis et pupillae*). The disease is due to recessive mutations in the ADAMTSL4 gene (1q21.2) and dominant mutations in the FBN1 gene (15q21.1). By definition, patients with IEL have no associated systemic abnormalities, although cardiac and skeletal examinations should be performed to help exclude Marfan syndrome. **Objective**: This report describes a case of a patient with crystalline subluxation and other ocular anomalies and suspicion of Marfan syndrome, which is finally diagnosed of IEL by NGS sequencing. **Case report:** 5 years female patien, without family history of interest. Size: 116.1cm (p76, 0.71 SD), weight: 16.9kg (p18, -0.92 SD), BMI: 12.54% (p7, -1.54 SD), body surface: 0.74m2, Tanner 1. Presents, in the right eye : superior subluxation of the lens with coloboma of the same, phacodonesis (lens *tremor*), amaurosis (total blindness), endotropia (deviation of the eye) intermittent to + 10º; left eye: superior subluxation of the lens, phacodonesis, decreased visual acuity (0.5 PIGASSOU). The electrocardiogram and Doppler-echocardiogram showed no pathology. **Methods**: NGS sequencing of the coding regions of the FBN1, TGFBR1 and TGFBR2 genes was performed: DNA amplified by multiplex PCR with the Ion *AmpliSeq* kit, *Ion Torrent* platform sequencing, bioinformatic analysis using *Thermo Fisher Scientific Variant Reporter*, with an average coverage obtained from 616.987X, covering 96.03% of the coding regions at 20X by massive sequencing and the rest by capillary sequencing. **Results**: A variant of uncertain clinical significance c.6801C> A (p.Asn2267Lys) is detected in heterozygosis in exon 56 of the FBN1 gene (sense change mutation). In order to determine if this change is inherited or *de novo*, genetic study was done to the parents and the brother, who didnt´t present the variant. **Conclusions**: This alteration has not been previously described in the literature, so it´s considered of unknown clinical significance. However, several bioinformatic tools specialized in the prediction of the effect of mutations (*Polyphen, SIFT, MutationTaster*) classify it as deleterious. Compatible *in silico* prediction, the fact the the variant found in our patient is *de novo* and the pathophysiology context of the FBN1 gene alterations and the proteins it encodes, we assume that the mutation c.6801C> A (p.Asn2267Lys) of the FBN1 gene is responsible for the diagnosis of isolated ectopia lentis.

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End-to-end automated workflow for simultaneously genotyping of multiple clinically relevant single nucleotide polymorphisms

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Background: Traditionally, the molecular pathology laboratory relies on manual techniques. Especially, when laboratory-develop tests (LDT) or tests of low demand are considered (e.g., genotyping of clinically relevant nucleotide variations of the human genome). However, end-to-end automated solutions for these tests are now available leading a paradigm shift in the field. Thus, the present study aimed to validate an automated and laboratory information system (LIS) integrated qPCR workflow for simultaneous detection of seven clinically relevant single nucleotide polymorphisms. **Methods:** This automated workflow validation included EDTA-whole blood from volunteers with known results for factor V Leiden (n=239), G20210A mutation in prothrombin gene (n=154), HLAB-27 allele status (n=235), C−13910T mutation in lactase gene (n=73), C677T mutation on methylenetetrahydrofolate reductase gene (MTHFR) (n= 99) and C282Y and H63D mutation in hemochromatosis gene (HFE) (n=52). The complete automation workflow was provided by the flow classic solution (Roche). The platform consisted of two automated liquid hander workstations (ALHW) (one for primary samples aliquoting and the other for qPCR set up), an automated nucleic acid extractor (Magna Pure 96) and a 384-well thermocycler (Light Cycler 480II). During the workflow, the instruments integrator software receives a sample worklist from the LIS (including samples barcode and the test to be performed). The first ALHW identifies the samples introduced by the operator allowing the creation of worklists for the downstream instruments and transfers 50ul of whole blood for an extraction plate. Genomic DNA is purified by the automated nucleic acid extractor. Simultaneously, the PCR reactions are set up by the second ALHW. For that, the instrument is loaded with sybr green qPCR master mix, ARMS qPCR primers (n= 21) and empty tubes for the allele-specific reaction mixture preparation (n=14) (according to with the worklist provided by the first ALHW). After the reaction mixture preparation and distribution into the qPCR plates, the extracted DNA and controls DNA (comprising all possible genotypes) are loaded into the equipment and transferred their specific qPCR wells. After the thermocycling, the amplification data is transferred to the integrator software, inspected by the operator together with the melting curves and the approved results are sent to LIS without any typing. The workflow can process 82 samples total (irrespective of the requested test) in 5 hours, have complete traceability, and for its validation, the obtained by results was compared with the expected results. **Results:** The automated workflow attributed the expected genotypes for all samples in all instances: 223 GG, 13 GA and 3 AA for factor V Leiden; 144 GG, 10 GA and 0 AA for Prothrombin G20210A; 29 positive and 206 negative for HLA-B27; 28 CC, 38 CT and 7 TT for Lactase C−13910T; MTHFR 43 CC, 46 CT, and 10 TT for MTHFR C677T and 48 GG, 3 GA, 1 AA for HFE C282Y and 35 CC, 14 CG, 3 GG HFE H63D mutation in hemochromatosis gene. **Conclusion:** The proposed automated qPCR workflow could accurately genotype seven distinct SNP. Its full automation confers higher safety and quality for the process.

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The serum has a higher yield of Janus kinase 2 V617F somatic mutation compared to the paired EDTA-whole blood sample.

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Background: Janus kinase 2 (JAK2) V617F is a somatic mutation of blood cells, usually detected in genomic DNA extracted from nucleated cells of the whole blood. As coagulation releases genomic DNA of these cells to serum, this specimen could be used as a source of DNA for the JAK2 V617F detection and quantification. This study aimed to test if JAK2 V617F can be detected reliably in serum and investigate if the percentage of the JAK2 mutant allele in serum is comparable to the rate found in whole blood. **Methods:** This study enrolled 88 subjects, 28 with positive for JAK2 V617F and 60 healthy volunteers. Paired EDTA-whole blood and serum samples were collected from each participant in two 4 mL tubes: EDTA-whole blood and Vacuette Z serum sep with clot activator (both from Greiner Bio-One). Genomic DNA was extracted from 500 ul of serum or 200 ul of EDTA-whole blood using Magna Pure 96 instrument (Roche) or EasyMAG (Biomerieux) according to the manufacturer's recommendations. EDTA-whole blood and serum extracted genomic DNA were quantified by a qPCR. The JAK2 wild-type (WT) and mutant (MUT) alleles were assessed by separated AS-qPCR reactions using the StepOne real-time system. The RNase P was co-amplified in both reactions to function as a normalizer gene. The percentage of JAK2 MUT allele was calculated by the delta-delta Cq method using JAK2 WT allele as comparator sample. The agreement between the rate of JAK2 mutation in EDTA-whole blood and serum was calculated using Pearson correlation and Bland-Altman analysis. The mean percentage of JAK2 MUT in paired specimens was compared by paired t-test. **Results:** Qualitatively, there was a complete concordance between serum and EDTA-whole blood results (28 positives and 60 negatives). Quantitatively, there was a correlation between serum and EDTA-whole blood results ($r=0.987$, $p<0.0001$). The JAK2 MUT yield bias between both specimens where -3.78% and the 95%CI agreement was -15.37% and 7.77%. The mean percentage of JAK2 MUT in serum was 33.89% (31.09) in serum and 30.11% (27.57) in the EDTA-whole blood ($p=0.0021$). **Conclusion:** JAK2 V617F mutation could be reliably detected in serum by qPCR using delta-delta Cq method. The JAK2 MUT allele burden in serum was significantly higher than in EDTA-whole blood. Serum results tended to be 3.78% higher than the EDTA-Whole blood results. This finding can be explored to increase the mutant allele detection rate in the routine.

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Simplified workflow for PML-RARA quantification in whole blood by automated nucleic acids extraction, multiplex one-step RT-qPCR and ΔΔCq method

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Background: Molecular testing for the PML-RARA fusion gene by RT-qPCR is a substantial approach for monitoring the response to therapy and predict relapse of patients with acute promyelocytic leukemia. The most frequent fusions subtypes are bcr1, bcr2 and bcr3 (found in ~55, ~5 and ~45% of the patients, respectively). The quantification assays for these transcripts have been standardized at international level. However, they are laborious and often require multiple steps that are not user-friendly. Thus, the objective of this study was to validate a simplified multiplex bcr1, bcr2 or bcr3 fusion quantification in whole blood and bone marrow by RT-qPCR. **Methods**: We used patient's specimens, or known amounts of synthetic fusion RNAs spiked into the negative samples. Red blood cells were removed from buffy coats prior extraction using PharmLyse (BD Biosciences). Nucleic acids were extracted on Magna Pure 96 (Roche). The reaction was designed to quantify bcr1/bcr2 or bcr3 and ABL1 RNA in a multiplexed one-step RT-qPCR reaction performed with QuantiNova RT-qPCR master mix (Qiagen) on LightCyclers 480II or z480 (Roche). Europe Against Cancer primers/probes for bcr1, bcr3, and ABL1 and Chen et al. 2015 for bcr2 were used. Ipsogen's (Qiagen) standard curves were used for the absolute quantification of bcr1/bcr2, bcr3, and ABL1. The applicability of the ΔΔCq method was evaluated analyzing the amplification efficiencies retrieved from a serial dilution of the synthetic RNAs corresponding to the studied molecular targets. The limit of detection (LoD) was determined by using probit regression analysis to a serial dilution of known amounts of each target. Precision was evaluated by CLSI EP12-A2 method on samples spiked with the high, medium and low amount of targets RNA in triplicate for seven days. The accuracy was assessed with the spike-recovery method by using Pearson correlation coefficient (r) and Bland-Altman analysis. **Results**: The median (Max-Min) of ABL1 achieved by the proposed workflow was $9.6x10^4$ ($3.98x10^3$-$7.11x10^5$) copies/sample. bcr1/bcr2 or bcr3 and ABL1 RT-qPCR efficiencies did not differ significantly in all tested occasions (95.18% for bcr1/bcr2; 97.95% for bcr3; 98.6% for ABL1) meaning that the ΔΔCq method is applicable. The LoD were 0.026% (95%CI 0.015-0.078) and 0.014% (95%CI 0.010-0.031) of PML-RARA/ABL1 for bcr1/bcr2 and bcr3, respectively. Imprecision values for low, medium and high levels of fusion synthetic RNAs, expressed as CV(%), were 4.98%, 10.65% and 11.77% for bcr1 and 5.03%, 9.56% and 27.46% for bcr3. In The Spike-recovery experiment, expected and observed measurements revealed a correlation coefficient (r) of 1.0 for bcr1/bcr2 and bcr3, and bias of 0.011 Log for bcr1/bcr2 and 0.035 Log for bcr3. Cross-reactivity between bcr1/bcr2 and bcr3 assays was not observed. **Conclusion**: We described a reliable and user-friendly workflow for PML-RARA bcr1/bcr2 and bcr3 fusion quantification in whole blood and bone marrow that reduces the number of steps proposed by the current guidelines. The proposed workflow showed acceptable sensitivity, precision, and accuracy. The assay reached the sensitivity at least 4.4-Log reduction based on the bcr1 and bcr3 positive samples. The main drawback in this study is the lack of bcr2 patients' samples.

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Rapid Somatic Mutation Testing in Colorectal Cancer Using a Fully Automated System and Single-Use Cartridge: A Comparison with Next-Generation Sequencing

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Background: Molecular tests have been increasingly used in the management of various cancers as more targeted therapies are becoming available as treatment options. The Idylla™ system (Biocartis, Mechelen, Belgium) is a fully integrated, cartridge-based platform that provides automated sample processing (deparaffinization, tissue digestion and DNA extraction) and real-time PCR-based mutation detection with all reagents included in a single-use cartridge. **Objective:** This retrospective study aimed at evaluating the Idylla™ KRAS and NRAS-BRAF-EGFR492 Mutation Assay cartridges against next-generation sequencing (NGS) using colorectal cancer (CRC) tissue samples. **Methods:** Forty-four archived formalin-fixed paraffin-embedded (FFPE) CRC tissue samples previously analyzed by targeted NGS were tested on the Idylla™ system. Among these samples, 17 had a mutation in *KRAS,* 5 in *NRAS,* and 12 in *BRAF* as determined using the Ion AmpliSeq 50-gene Cancer Hotspot Panel v2 (Thermo Fisher Scientific) . The remaining 10 samples were wild-type for *KRAS*, *NRAS* and *BRAF*. Tow 10 µm FFPE tissue sections were used for each Idylla™ run, one for the KRAS cartridge and one for the NRAS-BRAF-EGFR492 cartridge. All cases met the Idylla™ minimum tumor content requirement for *KRAS, NRAS*, and *BRAF* (≥ 10%). Assay reproducibility was evaluated by testing commercial standards derived from human cell lines, which had an allelic frequency of 50% and were run in triplicate. **Results:** The Idylla™ system successfully detected all mutations previously identified by NGS in *KRAS* (G12C, G12D, G12V, G13D, Q61K, Q61R, A146T), *NRAS* (G12V, G13R, Q61H), and *BRAF* (V600E). Compared to NGS, Idylla™ had a sensitivity of 100% (CI 90 – 100%). No mutations in the wild-type samples were detected by Idylla™, except for one sample that showed an *NRAS* mutation upon initial testing. Idylla™ testing was repeated twice on additional sections from this sample and it was negative. The negative result was further confirmed by another NGS method. Analysis of the horizon mutated control samples demonstrated agreement with the expected result for all samples and 100% reproducibility. The Idylla™ system produced results quickly with a turnaround time of approximately 2 hours. For certain mutations, Idylla™ did not distinguish between mutations occurring in the same codon (e.g. p.Q61R/L in *KRAS*, G12A/V in *NRAS*, and V600E/D in *BRAF*). **Conclusion:** The fully automated Idylla™ system offers reliable and sensitive testing of clinically actionable mutations in *KRAS, NRAS* and *BRAF* directly from FFPE tissue sections. Its simplicity and ease of use compared to other available molecular techniques make it suitable for small centers that lack highly trained staff and molecular expertise. Additionally, it can complement NGS and other molecular testing systems at larger diagnostic centers by providing significantly faster turnaround times.

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Absolute Quantification Of Graft-Derived Cell-Free DNA As A Marker Of Rejection And Graft Injury In Kidney Transplantation - Results From A Prospective Observational Trial

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Background: Graft-derived cell-free DNA (GcfDNA), a promising new, noninvasive biomarker of allograft rejection and injury status, was investigated in renal transplant (RTx) patients. **Methods:** In a prospective observational trial, GcfDNA was evaluated at prespecified visits in 88 RTx patients, followed over at least one year post transplantation. Relative percentage (GcfDNA%) and absolute quantification of GcfDNA copies (GcfDNAcp/mL) were performed as previously described [Beck J et al, Clin Chem 2014; 60 (Suppl.): S194-S195]. Biopsies were obtained upon clinical suspicion of acute rejection. GcfDNA results were compared between patients with and without positive biopsies. Data were analyzed using R 3.4.3 (packages: base, nparcomp). Pearson correlations were performed on log transformed data. **Results:** In patients (N=30) without subsequent rejection, active infections, or interventions, GcfDNA was highly elevated (median: 367 cp/mL; 4.20%), presumably due to ischemia/re-perfusion damage, in day 1 post RTx samples (n=10). In all 30 patients GcfDNA values decreased over the first 30 days to a baseline median of 19 cp/mL (0.20%), where it remained throughout the one year observation period. In patients (N=14) with samples (n=21) drawn during biopsy-proven acute rejection (BPAR) periods, median GcfDNAcp/mL was 5-fold and median GcfDNA% 2.6-fold higher (86 cp/mL; 0.68% respectively) than the medians observed in samples (n=267) from 62 clinically stable patients without rejection (17 cp/mL; 0.26%). These comparisons were confirmed by GcfDNA medians in 5 patients with negative biopsies (14 cp/mL; 0.18%). Both GcfDNAcp/mL and GcfDNA% were significantly different between patients with BPAR and apparently stable patients ($p<0.0001$). To compare the diagnostic accuracy of GcfDNAcp/mL and GcfDNA%, the area under the ROC curves (AUC) were calculated in 76 patients. GcfDNAcp/mL (0.88, 95% CI: 0.82-0.92) better discriminated between patients with BPAR and clinically stable patients than did GcfDNA% (0.81, 95% CI: 0.73-0.87). Plasma creatinine was not an independent marker, as it was used clinically as an indication for biopsies. Youden-index (YI)-based diagnostic sensitivity was 90% for GcfDNAcp/ml, and 86% for GcfDNA% obtained from ROC curves. Diagnostic specificity was 76% for GcfDNAcp/mL, and 74% for GcfDNA%. The threshold at maximum YI was 37 for GcfDNAcp/mL, and 0.43 for GcfDNA%. The correlation between GcfDNAcp/mL and GcfDNA% was r=0.75. Creatinine showed a moderate correlation with GcfDNA (cp/mL: r=0.44; %: r=0.41). In a selected patient subgroup (N=25) without clinically suspected rejection and a change of tacrolimus concentrations >60%, in samples (n=78) collected at ≥3 consecutive visits there was a negative correlation (r=-0.49) between tacrolimus and GcfDNAcp/mL. This sug-

gests that GcfDNA may detect silent graft damage due to under-immunosuppression which might increase the risk of de novo DSA formation and subsequent graft loss. **Conclusion:** This is the first systematic comparison of GcfDNAcp/mL with GcfDNA%. Absolute GcfDNA quantification allowed for a better discrimination than GcfDNA% of RTx patients with acute rejection and graft injury, due to less influence of recipient cfDNA variations, and may facilitate personalized immunosuppression.

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Validation of pre-analytical procedures of liquid biopsy samples for investigation of the *EGFR*-T790M mutation by ddPCR in NSCLC Brazilian patients

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Background: Non-small cell lung cancer (NSCLC) represents approximately 85% of all lung cancer types with 5% of survival rate. The T790M mutation in *EGFR* gene accounts for nearly 50% of the acquired resistance to tyrosine-kinase inhibitors (TKIs). Circulating-free tumor DNA (cfDNA) harboring T790M can be detected even at low concentration in plasma. Pre-analytical best practices is mandatory to ensure optimum yield and quality of the cfDNA. **Objective**: The aim of this study it was to validate the pre-analytical steps and establish the analysis protocol of droplet digital PCR (ddPCR) for measuring the levels of T790M mutation in NSCLC liquid biopsy samples. **Methods**: Plasma obtained from 5 health volunteers and 13 commercial controls (Horizon) with different T790M concentrations it were used to determine the limit of detection (LOD) and limit of quantification (LOQ), respectively. Patient samples were collected from different regions of Brazil to demonstrate the clinical performance of the test. *Training Set:* it were collected blood samples in EDTA (4mL) and PAXgene Blood cfDNA (PreAnalytix) tubes (16 mL) from 5 NSCLC patients. *Validation Set:* blood samples from 38 NSCLC additional patients were collected in EDTA tubes (4mL). EDTA samples were centrifuged, plasma was aliquoted and sent frozen to our center. One aliquot (2mL) of each EDTA sample were processed for qPCR cobas® EGFR Mutation Test v2 (Roche). PAXgene tubes blood samples it were send in room temperature and processed 24hs, 72hs or 168hs after collection. cfDNA samples (EDTA and PAXgene) were collected using QIAamp MinElute Virus and QIAamp Circulating Nucleic Acid kits (both QIAGEN) and yield measured using Qubit 1.0 (ThermoFisher). All cfDNA samples were submitted to ddPCR using T790M ddPCR Mutation Assay (BioRad) and QX100 Droplet Digital PCR System (BioRad). **Results:** The false-positive rate it was 0.12% and LOD stablished was ≥5 positive droplets to call a T790M positive result (95% CI). The expected results for control samples were 0.05, 0.5 and 5% of mutant allele frequency and the observed results were 0.053, 0.63 and 5.4% (r^2=0.99) with mean LOQ of 0.57%. Among training set, cfDNA concentration did not show significant statistically difference regardless time-point processing (p=0,6489) and collection tubes (p=0,07). cfDNA yield it was 10 times higher after virus kit collection (mean 0,52 ng/uL) compared to circulating kit (mean 0,05 ng/uL). All these patients were negative for T790M by both PCR methodology, however 4/38 (10%) patients from validation set were positive by qPCR (SQI ≤5) and negative for ddPCR. **Conclusions**: cfDNA collection using virus kit was superior compared to circulating kit. Four patients from validation set (10%) showed discordant results. This could be explained due to the loss of stability of cfDNA from frozen plasma. In addition, recent data has shown inverse correlation between qPCR results with SQI ≤5 and ddPCR T790M negative results. Our data showed that ddPCR is a powerful methodology for detection of low levels of T790M in NSCLC patients, however pre-analytical best practices is crucial for cfDNA stability and ddPCR sensitivity in liquid biopsy samples. COBAS qPCR results with SQI ≤5 should be revised.