Wednesday, July 29, 2015

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

Molecular Pathology/Probes

B-175

Study of Patatin-Like Phospholipase-3/Adiponutrin I148M Polymorphism and Biochemical Markers in Nonalcoholic Fatty Liver Disease

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Background: Nonalcoholic fatty liver disease (NAFLD) is the most common disease associated with abnormal liver function test. Its diagnosis requires the demonstration of fat in liver by either imaging study or liver biopsy. Some gene polymorphisms have been reported to be associated with NAFLD in a number of studies. We hypothesize that the single-nucleotide polymorphism rs738409 C>G at position 148 in the patatin-like phospholipase-3 gene (PNPLA3) will increase the susceptibility of NAFLD. This study aims to examine the association of PNPLA3 polymorphism with the prevalence and severity of NAFLD among Kuwaiti lipid clinic patients and to study the association of NAFLD with different markers of liver damage including alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total bilirubin, and tissue inhibitor of metalloproteinases-1 (TIMP-1). Method: 137 Kuwaiti lipid clinic patients (99 cases and 38 controls) were enrolled. The main inclusion criterion was attendance at the lipid clinic, and the main exclusion criteria were evidence of other causes of liver disease. Anthropometric parameters, general biochemistry profile, full lipid profile, TIMP-1, PNPLA3 gene and abdominal ultrasound were analyzed. Statistical analysis were performed including linear and binary logistic regression analysis which was used to ascertain the association of variables with the NAFLD and metabolic syndrome as dependent variables with and without adjustment for the confounding effects of age and waist circumference. Mean values were compared by Kruskal-Wallis one-way ANOVA test. Spearman correlation coefficient was used to describe the association between TIMP-1 and other variables of interest. A p-value < 0.05 was considered as statistically significant. Results: The I148M variant of PNPLA3 gene and markers of liver damage including ALT, ALP, GGT, total bilirubin, and TIMP-1 were not associated with NAFLD in Kuwaiti lipid clinic patients. NAFLD patients were older ($p < 0.001$), had significantly increased body mass index (BMI) ($p = 0.002$), waist circumference (WC) ($p = 0.004$), hip circumference (HC) ($p = 0.007$), waist-hip ratio ($p = 0.017$), fasting blood glucose ($p < 0.001$) and triglyceride (TG) ($p = 0.002$) and lower high density lipoprotein-cholesterol (HDL-C) ($p = 0.046$) in comparison to control group. Diabetes mellitus showed significant association with NAFLD (odds ratio [OR], 4.66; 95% confidence interval [CI], 1.68-12.97; $p = 0.002$). Moderate-to-severe ultrasound-defined hepatic steatosis was significantly associated with older age ($p = 0.034$), increased BMI ($p = 0.045$), WC ($p = 0.044$), HC ($p = 0.046$), TG ($p = 0.002$), ALT ($p = 0.026$), and low HDL-C ($p = 0.002$). Severity of steatosis showed significant association with metabolic syndrome ([OR], 2.77; 95%[CI], 1.35-5.71; $p = 0.005$). Patients carrying the CG and GG genotypes had significantly higher levels of total bilirubin ($p = 0.043$) compared to those carrying the CC genotype. TIMP-1 showed direct correlation with ALP ($r = 0.23$) and inverse correlation with total cholesterol ($r = -0.24$) and low density lipoprotein-cholesterol ($r = -0.19$). Conclusion: Unlike reports from other populations, the I148M variant of PNPLA3 gene and biochemical markers of liver damage including ALT, ALP, GGT, total bilirubin, and TIMP-1 are not associated with NAFLD in Kuwaiti lipid clinic patients.

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Rapid Screening for Targeted Genetic Variants via High-Resolution Melting Curve Analysis

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Background: Appropriate dosing of pharmaceuticals is critical to prevent sub-therapeutic efficacy or toxicity. Genetic variability in drug-metabolizing enzymes, including members of the cytochrome P450 (CYP450) superfamily, may significantly alter drug pharmacokinetics. CYP450 family members, including CYP2C9, CYP2C19, and CYP2D6, are responsible for the metabolism of >75% of commonly used therapeutic agents. Thus, targeted genetic screening for single nucleotide polymorphisms (SNPs) associated with aberrant enzyme function may considerably improve therapeutic regimens. In this study, we describe the validation of a method for the rapid screening for non-wild-type genotypes for targeted genetic polymorphisms in the CYP2C9, CYP2C19, and CYP2D6 enzymes via high-resolution melting curve (HRM) analysis. The method can rapidly identify individuals with non-wild-type sequences who should be further investigated for aberrant cytochrome P450 enzymatic activity.

Methods: DNA containing wild-type sequences of targeted SNPs for *CYP2C9*, *CYP2C19,* and *CYP2D6* were acquired from Coriell Institute. Sequence-specific primers were designed to amplify regions flanking targeted SNPs for *CYP2C9 (*2*,**3*), *CYP2C19 (*2*, **3*, **17*), and *CYP2*D6 *(*2*, **10*) and acquired from Integrated DNA Technologies. Polymerase chain reaction (PCR) parameters were optimized and performed under the same conditions for all SNPs: 5 min hold at 95°C followed by 5 s at 58°C and 7 s at 72°C for 40 cycles. Post-amplification, HRM analysis was performed over a temperature gradient of 65°C to 95°C with a 0.1°C step function. Development and validation studies were conducted on the Rotor-Gene Q (QIAGEN) thermocycler. Melting temperatures for each control were defined by the Rotor-Gene software as the midpoint of the melt phase, and data analyses were performed in Microsoft Excel. The multiplexed assay was validated through the assessment of primer specificity, intra- (n=5) and inter-assay (n=20) precision of melting temperatures, dynamic range analysis of DNA input concentrations, and concordance with Sanger sequencing. Validation studies were performed on wild-type, heterozygote and homozygote DNA templates. The entire assay run time for PCR amplification and HRM analysis is <2 h.

Results: Sequence-specific primers successfully amplified DNA amplicons of interest for targeted *CYP2C9*, *CYP2C19*, and *CYP2D6* regions, as determined by DNA gel analysis and classical Sanger chain-termination sequencing. Identical PCR amplification and HRM parameters were optimized and implemented for the screening of all aforementioned polymorphisms. Intra-assay precision of melting temperatures for all SNPs was ≤ 0.09%, while inter-assay precision of melting temperatures ranged from 0.04% to 0.21%. Further, upon normalization to wild-type sequences, melting temperatures of heterozygotes differed from wild-type melting temperatures by 0.12% to 0.33% for *CYP2C9*, 0.21 to 0.41% for *CYP2C19* and 0.13 to 0.15% for *CYP2D6* ($p < 0.05$). HRM screening for all SNPs displayed thermostability over a wide range of input DNA concentrations (10-200 ng/µl). Slopes of resulting melting temperatures versus input DNA concentrations did not exceed 0.0012 °C/(ng/µl), indicating that results were constant over all DNA concentrations.

Conclusion: The presented assay provides a high-throughput method for the rapid screening for genetic variants in targeted regions of the *CYP2C9, CYP2C19, and CYP2D6* genes. This method can be used to screen samples prior to targeted, probe-based confirmatory testing.

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The comparative Cq method can be used in the quantitative assessment of JAK2 V617F mutation by allele-specific qPCR in whole blood: no requirement for standard curves.

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Background:

The diagnostic value of JAK2 V617F mutation in myeloproliferative neoplasms is well established. The most widely used detection method involves allele-specific (AS) qPCR. This method also allows for quantification of JAK2 Wild-type (WT) and mutant (MUT) alleles percentages in the sample, generally, by using standard curves. However, the unique difference between WT and MUT AS-qPCR is the first nucleotide of the AS primers. It means that both reactions should have similar efficiencies and the ΔΔCq method could be applied for relative quantification of JAK2 alleles. The aim of the present study was to prove this hypothesis.

Methods:

This study enrolled whole blood samples (EDTA) from 27 healthy volunteers and 117 patients with known JAK2 V617F status (50 positive and 67 negative). Genomic DNA was extracted by using easyMAG (Biomerieux) and was quantified by a qPCR. The JAK2 WT and MUT were assessed by separated AS-qPCR reactions. 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 ΔΔCq method using JAK2 WT allele as comparator sample. The WT and MUT AS-qPCR efficiencies were evaluated by serial dilution of DNA samples with different JAK2 MUT levels. The assay linearity was determined by testing selected samples (JAK2 MUT from 0.5 to 99.69%). The lower limit of detection (LOD) was determined by probit regression analysis (JAK2 MUT 1:2 dilutions from 1.2 to 0.01%). For assay precision, the one-per-day run method (CLSI EP5-A2) and samples with 93%, 54% and 2,5% of JAK2 MUT were used. The accuracy was evaluated comparing the agreement between ΔΔCq method and ipsogen JAK2 MutaQuant kit (Qiagen).

Results:

The JAK2 WT and MUT AS-qPCR reactions showed similar efficiencies in all tested concentrations. The assay presented a linear response from 1 to 99.96% of JAK2 MUT allele. The LOD for the assay was 0.2% (95%IC 0.15-0.52%). The within run, between-run and total CV% were 0.24, 0.26 and 0.4% for the 93% of JAK2 MUT sample, 1.53, 1.14 and 1.9% for the 54% of JAK2 MUT sample and 6.21, 9.22 and 11.11% for the 2.5% of JAK2 MUT sample, respectively. The agreement with ipsogen JAK2 MutaQuant kit was high (R^2=0.997). The JAK2 MUT signal was observed in 50 out of 50 positive samples, in 0 out 67 negative samples and in 0 out of 27 healthy volunteers.

Conclusion:

The proposed ΔΔCq method along with AS-qPCR can be used for JAK2 V617F mutation detection and quantification in whole blood without the use of standard curves.

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Differentially expressed miRNAs are potential biomarkers for poor response to dual antiplatelet therapy in patients with coronary arterial disease

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Background: The variability of response to antiplatelet therapy depends on genetic background and environmental influence. Gene expression is regulated by pre- and post-transcriptional factors, including non-coding RNAs such as miRNAs. MiRNA profile has been proposed as important circulating biomarker for disease and therapeutic control. This study may contribute to prognosis of inadequate response to dual antiplatelet therapy in patients with coronary arterial disease (CAD). **Objective**: To investigate potential biomarkers of poor response to dual antiplatelet therapy by miRNome analysis in peripheral blood cells of patients with CAD. **Methods**: One-hundred-eleven CAD patients were selected, aged 30-70 years, under 100 mg aspirin and 300 mg clopidogrel loading dose 24 h prior to angioplasty. Platelet reactivity was measured by electrical impedance using ASPI and ADP antiaggregation tests. Patients in the low (T1) and high (T3) tertiles of aggregation units were considered responders and poor-responders to dual antiplatelet therapy, respectively. Six patients from each T1 and T3 groups were selected and total miRNA was extracted from whole blood. The mirnome was analyzed by NGS. Two libraries from T1 and T3 miRNA pooled samples were loaded on separated single-read flow cells and sequenced. The trimmed sequences were counted on mirDeep2 software and mirnome profiles of both responders and poor responders were compared using Deseq2 Biocondutor pakcage. Targeting and pathway analysis of the differentially expressed miRNAs was performed with the Ingenuity® suite, using filters for cardiovascular and inflammatory disease. **Results**: Poor-responders presented augmented expression of 12 miRNAs while 4 miRNAs had lower expression compared to responders ($p<0.05$). Ingenuity analysis revealed that these miRNAs have relevant targets, which are involved in platelet aggregation, platelet activation and clopidogrel metabolism, such as thrombin, Rac-1, ITGA2, ITGB3 and paraoxonase-1. **Conclusion**: The results are suggestive that differential expression of miRNAs in peripheral blood cells may be associated with variable response to antiplatelet therapy in CAD patients, and mirnome could be used to early prognosis of inadequate response to aspirin and clopidrogrel and possibly prevent restenosis after angioplasty.

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Mutation Analysis by Next Generation Sequencing (NGS) in de-novo Acute Myeloid Leukemia (AML)

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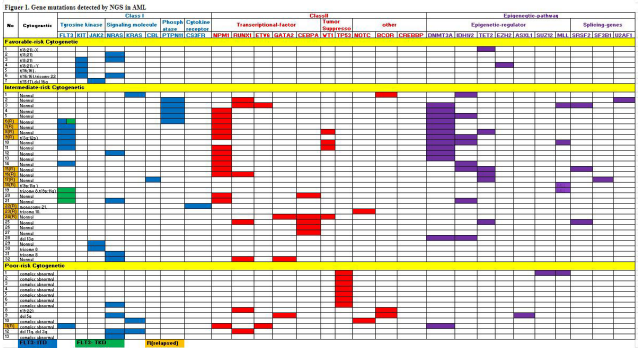
Background: Somatic mutations have been implicated in the pathogenesis of acute myeloid leukemia (AML). The classification and prognosis of AML increasingly depend on molecular and cytogenetic analysis. Mutation screening in AML is an integral part of risk stratification to guide therapy and monitor disease response/relapse. Next Generation Sequencing (NGS) can simultaneously detect multiple mutations in leukemia. We report mutations in AML using targeted NGS.

Methods: The study included 52 de novo AML from the pathology department/Knight diagnostic laboratory/Oregon Health and Science University (OHSU) (2013-2014). The targeted-NGS-panel covered 42 genes relevant to hematopoietic malignancies. Sequencing required 20ng of DNA from patients using Ion-Torrent-PGM. Bioinformatics-analysis was performed by Torrent-Suite-v.3.2 -pipeline (Life-Technologies, CA). Open-source programs and lab-developed algorithms were used for annotation and amino acid prediction.

Results: As shown in Figure 1, 100% of AML cases showed at least one mutation by NGS. In the intermediate-risk-cytogenetic-group, the most common mutations include transcriptional factors (NPM1, RUNX1, CEBPA) and epigenetic-regulators (DNMT3A, IDH, TET2, MLL) at the rate of 66% (21/32) for both. Mutations in splicing-genes (U2AF1, SRSF2, SF3B1) were less frequent (16%, 5/32). Multiple mutations (>2) were often seen (75%, 24/32); nearly all FLT3-ITD (100%, 7/7) or NPM1 (93%, 13/14) mutations occurred with epigenetic-regulators (DNMT3A, IDH and TET2). In the favorable-risk-cytogenetic-group, Kit (57%, 4/7) and Ras (43%, 3/7) mutations were common. In the poor-risk-cytogenetic-group, P53 (54%, 7/13) and Ras (38%, 5/13) mutations are frequent. Mutations with both DNMT3A and FLT3-ITD showed the high relapse rate (71%, 5/7).

Conclusion: AML is a biologically heterogeneous leukemia. Numerous recurrent mutations have been found and are used for risk stratification, targeted therapy and monitoring therapeutic response. This study demonstrates that targeted NGS mutation analysis is applicable in the clinical setting and provides a better understanding of leukemogenesis.



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Dengue virus serotypes distribution in a high-endemic country

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Background:

Dengue is an escalating health problem, predominantly in tropical areas where dengue viruses (DEN) can be transmitted. Four antigenically related but genetically distinct dengue viruses named DENV1, DENV2, DENV3 and DENV4 cause the disease. Since the late 1980's the incidence of dengue disease have increased in Brazil, a high endemic country where the majority of the cities are infested by *Aedes aegypti*. During the last 13 years, Brazil had four epidemics, with a total of about 4 million cases; in 2013 there were almost 1.5 million cases, which mark the highest of cases in this period.

Objective:

To evaluate the dengue virus serotype distribution from positive tests performed in a national base laboratory through the period from Jan 2012 to December 2014.

Methods:

Records from the laboratory were used to identify all positive tests obtained during the period of the study from all states of Brazil. The methodology for the tests were by reverse Transcription and Real-Time PCR (SYBR) followed by typing using Direct Sequencing.

Results:

Throughout the study period, the serotype dengue analysis was performed for 261 patients; 58 (22.2%) tested positive. DENV4 was the most frequent (33, 57%), followed by DENV1 (19, 33%), DENV2 (4, 7%), and DENV3 (2, 3%). The distribution varied according the year: DENV4 predominated in 2012 (57%) and 2013 (77%), whereas DENV1 predominate in 2014 (79%). The serotype distribution was similar between male and female, but changed according the age group.

Conclusion:

Our data are in concordance with the serotype surveillance data reported in Brazil. The co-circulation of multiple DENV serotypes and high dengue disease endemicity may be responsible for the increased distribution and severity of dengue disease in Brazil. Continuous monitoring of serotypes circulations is important to health public politics to control the dengue; the magnitude of this risk of epidemics also depends on the immunity levels established in each age group for each serotype and specifically the serotype that is more intensive circulating at each time.

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Enrichment of genomic DNA amount in serum by transport and storage at ambient temperature makes it an alternative matrix for molecular assays: high-yield of DNA, automated DNA extraction-friendly and direct use as template in qPCR.

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Background:

EDTA-whole blood is the matrix of choice for genomic DNA (gDNA) isolation for molecular testing. However, its automated DNA extraction has additional steps compared to serum/plasma and the presence of hemoglobin and EDTA impair its direct use as template for qPCR amplification. Curiously, the blood clot continually releases gDNA to serum ex-vivo. Thus, the aim of this study was to evaluate if this phenomenon can be explored in order to enrich the gDNA amount in serum making it an alternative matrix that surpass the whole blood drawbacks.

Methods:

Blood samples from fifteen healthy volunteers were drawn in Vacuette serum clot activator tubes (Greiner Bio-one). Five tubes were collected from each participant. The tubes were stored for 0h, 24h, 48h, 96h and 192h at room temperature before serum separation. The gDNA present in the serum was extracted by Easymag (Biomerieux). The extracted DNA or the crude serums were used as template for qPCR amplification of 65bp, 202bp, 440bp and 688bp amplicons. Next, blood samples from other fifteen volunteers were collected in the same above described tubes; they were shipped from Brasília (Federal District, Brazil) to Manaus (located in the Amazon rainforest) and then returned at ambient temperature. The samples came back after five days. The same above described qPCR was performed in these samples. Genotyping of hemochromatosis C282Y and H63D were also performed (amplicons of 102bp and 101bp, respectively). After that, crude serum (separated after five days at room temperature) from 21 consecutive patients from JAK2 V617F routine was used as template for qPCR. The results were compared to the standard assay performed on DNA extracted from EDTA-whole blood.

Results:

The median quantity of gDNA extracted from serum were 1.25, 2.03, 3.08, 10.1, 32.1µg/mL based on the 65bp PCR amplicon; 0.22, 0.41, 0.60, 2.6 and 9.4µg/mL based on the 100bp amplicon; 0.1, 0.25, 0.33, 1.44, 4.6µg/mL based on the 202bp amplicon and 0.001, 0.01, 0.02, 0.03, 1.8µg/mL based on the 688bp amplicon for 0h, 24h, 48h, 96h and 192h, respectively ($p<0.0001$ for 96h and 192h versus 0h, for all instances). Using the crude serum as template, the 65bp and 100bp amplicons were detected in all instances; the 202bp amplicon was detected only after 96h and the 688bp amplicon was not detected. In the samples shipped to Manaus, the median quantitative cycles for gDNA extracted from serum were 22.36, 23.61, 23.67 and 29.97 and for crude serum were 26.34, 29.34, 32.54 and not detected for amplicons of 65bp, 100bp, 202bp and 688pb, respectively. Furthermore, serum's gDNA and crude serum showed full concordance for hemochromatosis C282Y and H63D genotyping. Finally, crude serum and DNA extracted from whole blood showed complete agreement for JAK2 V617F mutation detection (17 negative and 4 positive).

Conclusions:

There is a significant increase of gDNA quantity in serum after 96h at room temperature that makes this specimen a source of high amount of gDNA compatible with rapid automated DNA extraction, with transport/storage at ambient temperature and with its direct use as template for amplification of small sizes' amplicons, except the DNA extraction.

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Primary drug resistance among HIV-1 recently infected patients in Brazil

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Background: According to the Brazilian Sexually Transmitted Diseases (STD) Program, the incidence of HIV is about 20 cases per 100.000 habitants. Genotyping test provides information about HIV-1 resistance to protease (PR) and transcriptase reverse (NRTI and NNRTI) class drugs. The goal of the government is to provide free and effective treatment to HIV patients but a new issue has arisen, the propagation of HIV-1 drug resistance strains among non-symptomatic individuals. This event called primary resistance can bring clinical and public health complications and may undermine HIV treatment with currently available drugs

Objective: To determine the prevalence of primary HIV-1 drug resistance in naive patients in a large private laboratory in Brazil.

Methods:We analyzed data from 782 plasma and blood samples genotyped for HIV-1 resistance mutations at the Molecular Diagnostics Laboratory (DASA) from the period of July 2014 to January 2015. Information about previous and current HIV treatment was taken from a patient's questionnaire completed prior to sample collection. Viral RNA was isolated using QIAamp RNA Viral Mini kit (Qiagen, Hilden, Germany). We amplified the protease gene and a fragment of reverse transcriptase from HIV-1 and sequenced in 3730 DNA Analyzer platform (Life Technologies, Foster City, CA). The calibrated population resistance tool (available through the Stanford University HIV Drug Resistance Database http://hivdb.stanford.edu) was used to identify the HIV subtypes and drug-resistances.

Results: From 782 samples, 145 (18%) naïve patients showed primary drug resistance or accessory mutations related to drug resistance, 32 patients (4%) presented primary drug resistance. From these 32 patients, 6 presented primary resistance to PR inhibitors and 25 primary resistance to NRTI and NNRTI´s class drugs. One patient presented primary resistance to all three classes of drugs.

The frequency of mutations mostly found in naïve patients were: K103N/S (NNRTI) 66.6%, M184I/V (NRTI) 15.1%, followed by D30N (PR) 12.1% and G48V / L90M (PR) 6%.

Conclusion: There are few studies about HIV-1 drug resistance in Brazil. The K103N is the most frequent mutation and confers resistance to Efavirenz, the first-line treatment in Brazil. The frequency of primary drug resistance was 4%, which is in accordance with five large studies in Brazil with rates from 2.2 to 8.1%.

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Increased Sensitivity of JAK2V617F Mutation Detection by COLD-PCR-based Sanger Sequencing

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Background: The point mutation of Janus kinase 2 V617F (JAK2V617F) has become a valuable marker for diagnosis of Philadelphia chromosome-negative chronic myeloproliferative neoplasms (MPNs) and an excellent target for therapeutic drug development. Although Sanger sequencing represents the criterion standard for characterization of the mutation, it is typically able to detect DNA variants present at moderate-to-high abundance, approximately 20% or greater. Co-amplification at lower denaturation temperature (COLD)-PCR has been reported as a powerful method to selectively amplify minority alleles from mixtures of wild type and mutation-containing sequences. In this study, we evaluated the efficiency of the combination of COLD-PCR and Sanger sequencing to detect JAK2V617F, in comparison with the conventional PCR-based Sanger sequencing.

Methods: Genomic DNA purified from cell line HEL with JAK2V617F mutation was diluted to 1%, 3%, 5%, 10%, 20% and 50% of JAK2V617F mutation DNA in wild-

type DNA from cell line K562. The DNA mixtures were amplified by fast COLD-PCR and conventional PCR, followed by Sanger sequencing on an ABI 3730XL DNA analyzer. Meanwhile, HEL cells was diluted to 1%, 3%, 5%, 10%, 20% and 50% of mutant cells in human healthy wild type whole blood cells, then genomic DNA was extracted with QIAamp DNA Blood Mini Kit from the cell mixtures. Then, the extracted DNA were amplified by the same COLD-PCR and conventional PCR protocols and sequenced. Furthermore, 114 JAK2V617F-positive DNA samples from patients were used to verify the established COLD-PCR-based Sanger sequencing method.

Results: The JAK2V617F mutation was detected in 20% mutant DNA mixture using conventional PCR and Sanger sequencing analysis, while not detected in less than 20%. However the mutation was clearly detected following fast COLD-PCR and Sanger sequencing even in 3% mutant DNA mixture. One the other hand, the JAK2V617F mutation was still detected in 20% mutant cells in wild type cells in whole blood matrix, not detected in less than 20%. By contrast, the mutation was clearly visible in 3% mutant cells in wild type blood cells using COLD-PCR based Sanger sequencing in a good accordance with the sensitivity observed in mutant DNA mixtures. Moreover, the JAK2V617F mutation was detected in 102 (89.37%) DNA samples from patients, not detected in 12 (10.63%) samples using conventional PCR based Sanger sequencing, while with COLD-PCR based Sanger sequencing analysis, the mutation was successfully detected in all 114 (100%) DNA samples from patients.

Conclusion: Compared to conventional PCR, COLD-PCR could greatly improve the sensitivity of Sanger sequencing method for JAK2V617F detection. COLD-PCR is powerful for mutation enrichment and mutation screening using clinical specimens with low-level mutations.

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Genotyping of the G6PD (Glucose-6-Phosphate Dehydrogenase) Gene: Applications in Pharmacogenomic Testing.

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Intro/Background:

G6PD deficiency is the most common human enzyme defect, estimated to be present in 400 million people worldwide. The *G6PD* gene is located on the X-chromosome. G6PD deficiency is inherited in an X-linked recessive manner therefore males are more commonly affected than females, but due to the high prevalence of G6PD deficiency, homozygous and compound heterozygous females are not uncommon.

G6PD is an important pharmacogene. With the exception of rare chronic nonspherocytic hemolytic anemia (CNSHA) related G6PD deficiency; individuals with G6PD deficiency are typically asymptomatic until an oxidative stress challenge occurs. Oxidative stresses, including those caused by many medications, can induce acute hemolytic anemia (AHA) in individuals who are G6PD deficient. AHA in G6PD deficient individuals can vary in severity, ranging from mild to life-threatening. The FDA recommends that patients at higher risk of G6PD deficiency, such as those with African or Mediterranean ancestry, be tested for G6PD deficiency before initiation of rasburicase therapy for hyperuricemia. *G6PD* genotyping is not widely available although enzyme testing is available. Our lab aims to make *G6PD* genotyping available to complement and, in some cases, replace G6PD enzyme testing.

Material/Methods:

A comprehensive genotyping of the *G6PD* gene was performed on six samples that had low G6PD levels (<0.3-6.4U/g Hb) by enzymatic assay, seven normals and a heterozygous deficient control. DNA was extracted from whole blood or saliva for analysis. A tagged-primer amplification method of all 13 exons, the intron-exon junctions, and 500 base pairs of the 3' untranslated region (UTR) followed by an enzymatic purification before performing bi-directional fluorescent dye-terminator sequencing, covering all previously described G6PD deficient variations. The sequencing was analyzed to determine variations using Mutation Surveyor™v.4.0.9 software.

Results:

In the sample set of fourteen, ten different single base variations were detected in the region of interest. Five of the variations detected result in amino acid changes, all classified as deleterious variations. All six samples selected for low G6PD levels had deleterious mutations found by sequencing. Four previously described WHO class III variations: A- (202)/Ferrara I, Mahidol, Seattle/Lodi/Modena/Ferrara II/Athens-like/Mexico, and Gaohe were seen in our initial deficient population. The lowest of the G6PD enzyme levels were associated with hemizygous variant males, while the heterozygous variant females exhibited deficient G6PD enzymes levels but not as severely suppressed. No deleterious variations were found in the normal samples sequenced.

Conclusions:

Comprehensive *G6PD* genotyping will allow clinicians to accurately characterize the G6PD status of an individual, impacting pharmacogenomics decision making.. This testing allows clinicians to employ pharmacogenomics to identify those at risk for an adverse drug reaction due to G6PD deficiency prior to prescribing drugs known to elicit an AHA reaction in G6PD deficient individuals. This testing also has applications to identify the genetic cause of G6PD deficiency, infants at increased risk of neonatal hyperbilirubinemia, determine G6PD status in individuals with inconclusive/abnormal phenotyping results, carrier status in women, and classify patients experiencing CNSHA.

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Micro-RNA Signatures In The Vitreous And Plasma Of Patients Suffering From Exudative AMD

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Purpose: Age-related macular degeneration (AMD) is the leading cause of blindness affecting people over the age of 50. The exudative form, known as neovascular AMD (NV AMD), is characterized by choroidal neo-vascularization (CNV) that consists of abnormal leaky choroidal vessel growth into the retina. Recently, a number of studies have demonstrated specific micro-RNA (miRNA) signatures in plasma or urine for several diseases such as cancers and kidney disease. MiRNAs are small non-coding RNA of 22-24 nucleotides length which can circulate in human body fluids. To date, there is a lack of information on miRNA signatures associated with NV AMD. The aim of this study is to detect miRNA profiles in the vitreous and plasma of patients with NV AMD.

Methods: All patients were previously diagnosed with AMD (with CNV) and were followed and operated by a single vitreoretinal surgeon (F.A. Rezende) before receiving anti-VEGF treatment (AVASTIN). Control patients underwent surgical treatment for nonvascular pathology (epiretinal membrane or macular hole) by the same surgeon. In addition, for plasma miRNA detection, blood was collected the same day prior to the surgery. First, a screening of miRNAs in vitreous samples (3 controls and 4 AMD) was perfomed by microarray (including 384 miRNAs). Next, we validated miRNA profiles in both vitreous and plasma with individual TaqMan qPCRs with a higher number of patients (n=15) in each group.

Results: Microarray analysis identified significantly higher levels of miR-548a (2-fold) and miR-146a (4-fold) and lower levels of miR-16, miR-152 and miR-106b in human vitreous from patients with NV AMD when compared to controls. Individual qPCRs validated vitreous expression patterns of miR-146a, miR-106b and miR-152. In addition, analysis of plasma miRNAs by TaqMan miRNA assay identified a decrease in the level of miR-152 and miR-106b. In contrast, plasma levels of miR-146a were not significantly induced in the AMD group as they were for vitreous samples. Importantly, no correlation was drawn for miRNA profiles and patient age.

Conclusion: To our knowledge, our study is the first to characterize miRNA profiles in both vitreous and plasma from a cohort of patients suffering from NV AMD. Identification of a miRNA signature in circulation is conceptually promising for future development of a novel class of biomarkers for NV AMD. Moreover, elucidating miRNA profiles may provide insight on gene regulation during disease progression and potentially provide novel therapeutic avenues.

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MELPA: a Novel Technology for High-throughput, Multiplex Genotyping Directly from Dried Blood Spot without DNA Extraction, with an Application in the Screening of Multiple G6PD Gene Variants at Risk for Drug-induced Hemolysis

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Background: Hundreds of thousands of single nucleotide polymorphisms (SNPs) have been found to be associated with disease susceptibility, drug response and complex phenotypes. Clinical population screening of significant SNP markers calls for multiplexed genotyping of SNPs on a large scale. Current SNP genotyping tools

(e.g, TaqMan and microarray-based assays), despite many advantages, invariably require DNA extraction, which remains a key throughput-limiting step for population screening, and is technically challenging for small-volume precious specimens or long-term stored archived collections. In addition, multiplex-PCR amplification employed by these genotyping methods suffers from complex primer design and/or amplification bias. Here, we describe a novel high-throughput genotyping approach, Multiplex Extension and Ligation-based Probe Amplification (MELPA), which has multiplex SNP genotyping capability, eliminates DNA extraction, achieves uniform PCR amplification using a single pair of universal primers, and is suitable for archival Dried Blood Spot (DBS) samples.

Methods: Instead of nucleic acid extraction, MELPA lysed samples and captured the target DNA directly to 96-well plate by sandwich hybridization using multiple oligo probes with universal tail sequences. After enzymatic extension and ligation of the probes, a single-stranded template for each target SNP site was formed, and all templates were PCR-amplified using universal primers targeting the tail sequences. Multiplexed genotyping by single-base primer extensions were analyzed with a MALDI-TOF mass spectrometry platform (MassARRAY by Sequenom). We tested the feasibility of the new assay for whole blood and DBS samples, and evaluated the accuracy by comparing MELPA with both a commercial multiplex SNP assay (iPLEX) and DNA sequencing, for the detection of 23 G6PD gene variants known to be at risk for primaquine-induced hemolysis in antimalarial therapy. Finally, we employed MELPA in a G6PD variant preemptive screening of DBS samples from malaria-endemic areas.

Results: MELPA can be successfully applied on fresh or archival blood and DBS samples, with call rates >97%. G6PD genotyping by MELPA on 2 randomly chosen archival blood samples gave results more accurate than the iPLEX assay, and were 100% concordant with DNA sequencing. We conducted MELPA genotyping of 106 archival blood samples of *P. vivax* malaria patients taking primaquine and found 10 G6PD-deficient variants from 9 cases, including one hemizygous male patient with G6PD *Mahidol* mutation who had hemolysis. A preemptive G6PD genotyping of 438 DBS samples from a malaria-endemic area in Yunnan Province was conducted using MELPA, and 3 G6PD variants were identified. A sample-pooling strategy can further increase the sample throughput of the MELPA assay; elevated wild type background introduced by sample pooling can be reduced by adding specific wild-type allele blockers.

Conclusion: MELPA represents an efficient and cost-effective approach to multiplex SNP genotyping at population level. Preemptive genotyping of G6PD variants in high-risk population in malaria endemic areas presents a good paradigm for high volume epidemiological surveillance, and will be valuable for assessing drug-induced hemolytic risk for the development of optimal malaria therapeutic strategy for an endemic area.

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Whole-exome sequencing as a powerful diagnostic tool in clinical laboratories

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One of the major concerns of medical sciences is finding the causal genes underlying human diseases. In the last decade, great efforts were applied in the development of new molecular technologies, such as next generation sequencing and oligonucleotide arrays, aiming to elucidate the genetic basis of disorders. In this sense, with the advent of whole-exome sequencing (WES), identification of disease-causing genetic variations is progressing at rapid pace, improving the disease management by either available treatments or genetic counseling and risk assessment of patients andrelatives. In Hermes Pardini Institute, one of the largest clinical and diagnosis laboratory from Brazil, began offering this molecular test in 2014 and since then has enabled the genetic diagnose of many diseases. Here we report an overview of the WES use in different clinical situations by our lab group. Coding exons from 20 Brazilian patients were captured by Illumina Nextera technology and sequenced in the Illumina HiSeq 2500 platform. Reads were aligned to the human genome reference (hg19/GRCh37) and variants were identified through bioinformatics analysis. WES identified probably disease-causing mutations in nine cases from different disorders: Encephalopathy (2 cases), Cognitive Delay with Epilepsy, GLUT1 Deficiency Syndrome 1 (GLUT1DS1), Epilepsy, Spinal Muscular Atrophy type 1 (SMA1), Leigh Syndrome (LS), Tay-Sachs Disease (TSD) and Cat Eye Syndrome (CES). From these, WES revealed 9 previously undescribed mutations (4 missense mutations, 2 deletions, 1 insertion, 1 splice donor site mutation and 1 frameshift with stop gained mutation) and 2 previously described mutations (1 missense mutation and 1 splice donor site mutation). From these 11mutations, 4 were classified as damaging mutations, 5 as probably damaging and 2 were variants of unknown clinical significance in genes related to the clinical phenotype, and thus, need to be further investigated. WES failedto identify disease-causing mutations in 11 patients and this could be due to many factors, such as polygenic diseases, structural variants, epigenetics changes, insufficient coverageof few exons in exome sequencing and intronic or mitochondrial mutations. In conclusion, WES is an efficient, sensitive, specific and cost-effective method for disorders difficult to diagnose and has important implications for genetic counselling and clinical services.

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Performance characteristics and comparison of two real-time PCR systems for hepatitis B virus DNA quantification.

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Background: Detection and accurate quantification of hepatitis B virus (HBV) DNA are essential tools for the diagnosis and management of chronic HBV infected patients undergoing antiviral treatment. The use of real-time PCR assays for HBV DNA quantification is strongly recommended. With several HBV DNA real time PCR quantification assays available, it is important to use the most efficient and effective testing system for accurate virological monitoring. In this study, we evaluated the performance characteristics and comparability of two HBV real time PCR quantification systems: Artus HBV real-time PCR assay on Step one Applied Biosystems analyzer with manual viral nucleic acid extraction using QIAamp DNA blood kit, and COBASAmpliPrep/COBAS TaqMan HBV Test, v2.0 with automated viral nucleic acid extraction.

Methods: We evaluated precision, bias and linearity (Upper and lower detection limits). Precision data had been collected using pooled serum of HBV positive patients; this pooled serum was very well mixed, divided into aliquots and stored at -20 ºC then analyzed in triplicates in different PCR runs. Bias was calculated from proficiency testing results (UK-NEQAS) as the difference between the measured and the intended results for the provided samples. Linearity was determined using serial dilutions of a high viral load sample, regression line analysis was done between the expected and the measured values to verify the analytical measurement range of each assay (Upper and lower detection limits). Method comparison was done between both assays and correlation coefficient was calculated.

Results: Results for precision study revealed an overall variance of 0.0410 log IU/ml (CV: 0.89 %) for Artus HBV real-time PCR assay and 0.078 log IU/ml (CV: 2.24%) for COBASAmpliPrep/COBAS TaqMan HBV Test, v2.0 . Average bias estimation using 4 proficiency testing samples (UK-NEQAS) showed an average bias of 5.36 % for Artus HBV real-time PCR and 5.64 % for COBASAmpliPrep/COBAS TaqMan HBV Test, v2.0 . The analytical measurement range for Artus HBV real-time PCR was found to be from 2.0 X 10^0 to 2.9X 10^8 IU/ml (Regression line analysis; slope:1, r^2:1) while that of COBASAmpliPrep/COBAS TaqMan HBV Test, v2.0 was found to be 2.0 X 10^1 - 2.4 X 10^7 IU/ml (Regression line analysis ; slope:0.99, r^2: 1). Method comparison between paired quantitative results of both assays showed strong correlation with r^2 0.99

Conclusion: The performance characteristics and the strong correlation between results of both Artus HBV real-time PCR with manual nucleic acid extraction using QIAamp DNA blood kit and COBASAmpliPrep/COBAS TaqMan HBV Test v2.0 with automated nucleic acid extraction assays; suggest that both assays can be used for management and therapeutic monitoring of chronic HBV infection.

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15q11.2 microdeletion syndrome

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Background: Comparative genomic hybridization to arrays (array-CGH) analyzes the human genome to detect gains and losses of genetic material associated with microdeletion and microduplication syndromes. Array-CGH detects the presence of microdeletions and microduplications that would be undetectable by conventional cytogenetic techniques.

Case report: Child two years old with neurological dysfunction that has delayed motor development. The constitutional karyotype and genetic study Prader-Willi were negatives. Chromosome analysis was performed by array-CGH, Human G3 used CGH Microarray SurePrint 400K (Agilent®), with an average spacing between probes of 5.3 Kb and 4.6 Kb for RefSeq genes. The reading was performed using microarray Microarray Scanner G2565CA (Agilent®) at a resolution of 3µm and analysis software results Cytogenomics v 2.0.6.0 (Agilent®). The results show a genomic male pattern with the formula: arr (1-22) x2 (XY) X1, (ISCN 2009). The heterozygous microdeletion on chromosome 15 was detected between breakpoints BP1 and BP2 (15q11.2) with genomic coordinates chr15: 18692865-20308073, including deletion of GOLGA6L6, GOLGA8C, BCL8, LOC646214, CXADRP2, POTEB, NF1P1, LOC727924, OR4M2, OR4N4, OR4N3P, LOC646396, GOLGA8DP, GOLGA6L1 genes.

Discussion: Recent studies suggest that this area is a genomic region of susceptibility to neurological dysfunction, including developmental delays, autistic features, behavioral disturbances, attention deficit hyperactivity disorder, and mild dysmorphic features, leading to a new 15q11.2 microdeletion syndrome and might be associated with the clinical history of this patient. Also, the 15q11.2 microdeletion syndrome has been associated with proximal esophageal atresia, distal tracheoesophageal fistula, congenital cataracts idiopathic, generalized epilepsy, schizophrenia, and Alzheimer's.

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Genotyping the PKLR gene (Pyruvate Kinase, Liver, Red Blood Cell) for Non-spherocytic Hemolytic Anemia

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Background: The *PKLR* gene encodes for two isoforms of the enzyme pyruvate Kinase (PK), a glycolytic enzyme that transfers phosphate groups from phosphoenolpyruvate (PEP) to ADP creating pyruvate and ATP. The two isoforms have different exon 1 sequences that cause tissue-specific expression, L (liver) and R (red blood cell). The second leading cause of Non-spherocytic Hemolytic anemia is a deficiency of the enzyme pyruvate kinase. Patients have a wide range of phenotypic expression of hemolysis ranging from minor effects to life-threatening neonatal situations requiring blood transfusions. Anemic patients that have inconclusive findings by enzyme assays could benefit from the characterization of the *PKLR* gene to aid in the diagnosis of PK deficiency. Carrier testing is also possible.

Methods: Genotyping of the *PKLR* gene was performed on samples that had low PK (pyruvate kinase) levels by enzymatic assays. The DNA was extracted from whole blood then genotyped using two different methods (*PKLR* Full gene and Deletion assay). The first methodology employed tagged-primer amplification of all 11 exons for isoform R and included exon 1 for isoform L, followed by an enzymatic purification before performing bi-directional fluorescent dye-terminator sequencing. The sequencing was analyzed to determine variations using Mutation Surveyor™v.4.0.9 software for visual inspection. The second methodology utilizes fragment detection of a single amplicon (~10kb) covering intron 2 through the 3-primed untranslated region (UTR) for the presence of a large deletion. The amplified fragment was then sized using the Agilent 2100 Bioanalyzer and visually verified to be within the acceptable range.

Results: The tagged sequencing assay detected the causative mutation in patients sequenced exhibiting a low PK level. The large deletion detection assay identified patients with a deletion. One pair of samples had one individual with a heterozygous large deletion and a family member with a homozygous large deletion. The two method approach was very beneficial in these two samples. Neither sample had a mutation detected by the tagged sequencing assay however the large deletion was detected by the fragment assay. Combining the results from both assays gave greater understanding of the patient's genotype.

Conclusion: The *PKLR* full gene and large deletion detection assay utilized two different methodologies thereby giving a clearer picture of the cause of anemia in patients with PK deficiency. Most causative mutations were found in the sequencing portion of the assay however for the patients with a large deletion present, this was detected only by the fragment detection assay. The combination of the two assays together created a more accurate genotype for the patients.

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Frequency of microdeletions of the Y chromosome in a routine laboratory in Brazil

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Background: Y chromosome microdeletions are the second most frequent genetic cause of genetic infertility in men following Klinefelter syndrome. Molecular studies have shown that mcrodeletions at Yq11 may represent the etiology factor in as many as 10 to 20% cases of idiopathic azoospermia or severe oligoszoospermia. Most of the deletions occur de novo and fall in 3 non overlapping regions, designated Azoospermia Factor Regions (AZF): AZFa, AZFb and AZFc, of which the distally located AZFc is the most frequently deleted. The molecular diagnosis of these deletions became an important diagnostic test to investigate spermatogenic failure.

Objective: The aim of this study was to evaluate the frequency of Y microdeletions in the routine of a private laboratory in Brazil.

Methods: From January to December 2014, 491 blood and sperm samples were isolated at the QIAsymphony Platform (QIAsymphony DNA mini kit - QIAgen). The amplification strategy consisted in a multiplex polymerase chain reaction (Multiplex PCR - QIAgen) targeting six regions (AZFa: sY84, sY86, AZFb: sY127, sY134 and AZFc: sY254, sY255). The multiplex-PCR products were detected at QIAxcel Systems (DNA Screening Kit - QIAgen). All steps were processed and validated at Molecular Diagnostics Laboratory (DASA). Information about infertility problems was taken from patient's questionnaire filled prior to sample collection.

Results: We detected 15 (3%) patients that presented microdeletions from a total of 491 samples. From these positive for microdeletion patients, 4 patients showed deletions at the AZFb (sY127, sY134) region , 8 showed deletions at the AZFc (sY254, sY255) region and 1 presented all regions deleted (AZFa, AZFb and AZFc).

Conclusion: Diagnosis of a microdeletion of the Y chromosome permits the cause of the patient's azoospermia/oligozoospermia to be established and to formulate a prognosis. Deletions in the AZFc region on Y chromosome are the most common cause of spermatogenic failure, which is in accordance with the results of this study.

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A New Universal Multiplex Digital PCR Method with Improved Precision for the Quantification of Donor Derived Graft cfDNA Traces.

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Background: Graft-derived cell-free DNA (GcfDNA) quantification is gaining high interest as biomarker for graft integrity after transplantation. Using high throughput sequencing (HTS)1 or digital PCR (dPCR)2, the quantification of informative SNPs in total cell-free plasma DNA (cfDNA) became feasible. Molecule counting as employed by both methods enables the detection of small changes in GcfDNA levels with higher precision than qPCR. However, both methods include a preamplification using adapters ligated to the DNA, which introduces non-systematic amplification variability. Here we present a preamplification-free, multiplexed dual dPCR assay approach yielding a low overall imprecision.

Methods: Informative SNP assays were selected for each patient as described2. Four-plex dPCRs were performed in separate reactions for graft and recipient. Molecule concentrations were calculated by Poisson statistics using molecule counts of four combined graft-specific dPCRs with 5-7µL sample volume and one recipient-specific reaction (1µL sample). Simulations were computed to assess the achievable total precision of dPCR quantifications with and without preamplification. These results were experimentally validated by direct comparison of both methods in 11 cfDNA pools of mixed normal and 19 transplant patient samples containing 0.5%-30% GcfDNA. Twenty-four different four-plex dPCRs were performed in 207 HTx patient samples.

Results: The ligation efficiency is the limiting step in preamplification and ranges from 40% to 85%. In silico simulations, considering this ligation variability and the low DNA amount, indicated that direct multiplex quantification is superior to dPCR with preamplification, despite 40-fold higher positive events in the latter. Consistently, the relative standard deviation (CV) in 11 cfDNA pools was 2.1-fold higher after preamplification. The CVs of the multiplex method in 19 LTx patient samples (mean:11%,SD:6%) were significantly lower than those obtained with preamplification (mean:28%,SD:15%, p=0.009). In 207 HTx patient samples with a

median total cfDNA concentration of 63,210cp/mL plasma (range: 2,415-2,102,861) the median GcfDNA was 0.57% (range: 0.02-12.1%) resulted in a median CV of 7.6% (range: 1.0-38.3). A CV of <30% was achieved in 98.5% of all patient samples and 90% yielded <20% CV; the GcfDNA in the latter group ranged from 0.03-12.1% and 21-43,530cp/mL plasma.

Conclusion: The reliable quantification of rare DNA moieties is technically challenging, especially in cfDNA, with the scarce total DNA amount. The use of dPCR or HTS eliminates a potential calibration bias by direct molecule counting. Due to the hypergeometric contribution to the total measurement error, the variability in preamplification is deteriorating the precision, especially if the number of targets is very low, as in GcfDNA in HTx. Since this is a random error, it cannot be eliminated and therefore, avoiding preamplification resulted in lower imprecision. The advantage of direct cfDNA molecule counting is the ability to assess the total error by the Poisson counting error. With the testing of four independent SNPs in one dPCR and the summation of five multiplex dPCRs into one final result the number of counted molecules is sufficiently high to quantify GcfDNA levels in HTx patients with high precision at lower costs compared to HTS based methods.

1)DeVlaminck et al. Sci.Trans.Med(2014);6:241ra77

2)Beck et al. Clin.Chem(2013);59:1732-41

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Early Detection of Rejection after Heart Transplantation by a Universal Digital PCR Method.

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Background: Acute allograft rejection (AR) is a major complication after heart transplantation (HTx). Current standard for early detection of rejection is percutaneous-transvenous endomyocardial biopsy (EMB), a procedure, which is burdening, associated with risk for serious complications and false negative results, which can occur due to the patchy nature of AR. The quantification of Graft derived cell-free DNA (GcfDNA) is reported as biomarker for graft integrity, which can early detect AR in solid organ transplantation, only requiring a simple blood draw. Aim of the study was to investigate the diagnostic use of GcfDNA during the first year after HTx.

Methods:30 Patients (23m,7f) undergoing HTx were included with an age range of 26 to 69 years, of which 26(81%) survived the first year. 16 samples were drawn per patient during the first year post Tx. Immunosuppression was based on CsA or Tacrolimus with Mycophenolate and 6 patients received an mTor inhibitor. EMB-proven AR occurred in 13 Patients, where two had a Grade 2 AR after more than 120 days post HTx. GcfDNA was measured with a modified published digital PCR method, based on an universal probe set. The data were calculated as percentage of graft cfDNA per total cfDNA and as absolute values in copies/mL (cp/mL) of plasma.

Results: Immediately after engraftment, the GcfDNA was high with a median of 4.3% (IQR:1.9-5.2) and 3,478cp/mL (IQR:1,733-8,172), and decreased with an approx. half-life of 3.3 days (%) and 4.5 days (cp/mL). In uncomplicated courses the levels after 2 weeks were below a threshold of 0.6% and 160cp/mL respectively (95thpercentile).

In the initial 2 weeks distinct increases in individual courses are better suited to indicate a rejection, since the GcfDNA and total cfDNA levels show high inter-individual variability during the post Tx decay period.

In two cases of late rejections, both at one year with samples at the time of EMB-proven AR levels of 3% (694cp/mL) and 11% GcfDNA (512cp/mL) were detected. In three other cases of EMB-proven AR the GcfDNA increased from a) 0.15% to 2.88%, where the first significant change was seen after 6 months and the rejection was diagnosed after 12 months. b) in one earlier EMB-proven AR (2 months) GcfDNA increased beginning at 1 month to 3% and was falling to 0.6% after successful treatment. And c) the third case had a subtle steady increase from 0.2 to 0.7% beginning 2 months before diagnosis of AR at 6 months; GcfDNA reverted to 0.2% after treatment.

Conclusion:A modified dPCR method without pre-amplification was used to quantify GcfDNA. The measurements can be done within one working day at reasonable costs. Compared to earlier observations in liver recipients, the post reperfusion phase is characterized by a longer recovery time of the graft as assessed by the GcfDNA half-life. During this phase increases of GcfDNA are subtle, but distinguishable during AR. After that period, rejections can be early distinguished by increasing and elevated GcfDNA levels, which in the later phase is characterized by early and sustained increasing levels up to 6 months before diagnosis of AR.

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Unusual mosaicism 45,X/47,XXX attenuates Turner's phenotype

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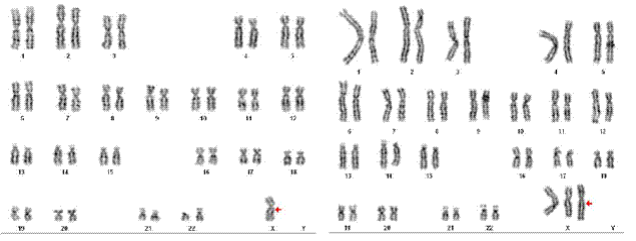
Introduction: Turner is syndrome characterized by ovarian failure, primary amenorrhea and short stature, it comprises a partial or complete absence of a sex chromosome and it's estimated that 1of 2.000 female newborns are affected. The ovarian failure generally occurs before puberty and leads to infertility. The triple X Syndrome has an incidence of 1 in 1.200 live born female and the phenotype varies greatly from women with normal intelligence and minimal dysmorphism to patients with severe mental disability. **Objective:** To describe an unusual karyotype finding with a mosaic for Turner Syndrome and Triple X Syndrome in a patient with Turner phenotype but without gonadal dysgenesis. **Method:** A 400-band Karyotype was performed in peripheral blood lymphocyte and 30 metaphases were analyzed in a 13 years old female patient to investigate short stature. The patient presented a suggestive phenotype for Turner Syndrome with 153 cm of height but with normal development of secondary sexual characteristics, menarche and a normal pelvic ultrasound. **Results:** The thirty metaphases analyzed 63% presented a monosomy of the sex chromosome X and 37 % a complete trisomy of the sex chromosome X: mos 45,X [19]/47,XXX [11] (Figure1). **Conclusion:** Mosaicism 45, X / 47, XXX is a very sporadic cause of ovarian dysgenesis. Many cases have been characterized by a variable Turner phenotype, including a 33 year old women with previous normal ultrasound, regular cycles and normal secondary sexual characteristic; at age 33 her ultrasound revealed evidence of streak-like ovaries, weight loss and breast regression. We might infer that this specific mosaic may attenuate the Turner phenotype enabling these women to have a menarche and normal pubertal development but the regression of the phenotype can occur in adulthood requiring constant monitoring and possibly a new karyotype to evaluate if the percentage of mosaicism remains the same.

45,X [19]/47,XXX [11]



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Mitochondrial DNA rare variants are genetic risk factors for type 2 diabetes for Chinese

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Abstract:**Background** The mitochondrial DNA variants and mitochondrial haplogroups have been suggested as risk factors for T2DM. However, the role of common variants, low frequency variants, rare variants, and singleton variants of mitochondrial genome with the risk of T2DM still have not been fully understood. Moreover, the interaction between haplogroups and variants in the mitochondrial DNA is also still clear. **Methods** In our studies, a total of 282 blood samples of type 2 diabetic patients and 396 blood samples of healthy controls were enrolled for analyzing from 2010 to 2013 in Zhejiang province, China.The entire mitochondrial genome for both patients and controls were sequenced. **Results** We demonstrated that the subhaplogroup M8a, included in mitochondrial haplogroup M, was associated with an increased risk of T2DM. While subhaplogroup N9a included in haplogroup N, was also associated with increased risk of diabetes. A total of 115 common variants (MAF≥5%), 288 low frequency variants, 599 rare variants (MAF<1%), and 835 singletons variants were identified across the entire mtDNA for the 2 major haplogroups M and N.For the common variants, our studies indicated that common variants in *CytB* were associated with decreased risk of diabetes only for haplogroup M. But the low frequency variants of mtDNA in both haplogroup M and N were not associated with risk of T2DM. In addition, the rare variants in *ND5* and *CytB* genes

might contribute to the elevation risk of diabetes in haplogroup M. In contrast, the rare variants in *ND6* and *16S* genes were associated with increased risk of diabetes in haplogroup N. **Conclusion** Take those results together, our studies may provide the novel genetic evidences of rare variants in mtDNA are crucial for increasing the risk of T2DM in Chinese.

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Performance Evaluation of New Solid Sample Types and Extraction Protocols on the Fully-Automated Tissue Preparation Solution

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BACKGROUND: Formalin-fixed, paraffin-embedded (FFPE) tissue samples are standard materials used by molecular pathologists, reference labs, research environments, and biobanks. An automated solution to extract high-quality nucleic acids from this challenging sample type is crucial to ensure accurate assay results and patient treatment. The IVD Tissue Preparation Solution (Siemens Healthcare Diagnostics, Tarrytown, NY), consisting of the Tissue Preparation System (TPS) and VERSANT® Tissue Preparation Reagents (TPR) Kit, is the only fully-automated method for deparaffinization and extraction of nucleic acids from FFPE samples with the proven flexibility to process a variety of sample type inputs as well. The new software delivers functionalities with expandable test definitions and an enhanced graphic user interface experience. The new software will include the existing *in-vitro* diagnostics (IVD) protocols (DNA, RNA, and SPLIT) as well as the new DNAext protocol. The new DNAext protocol leverages the validated workflow of DNA protocol while optimizing its extraction parameters to meet the increasing demand of novel molecular applications. The goal of this study was to evaluate the TPS protocols' extraction performance using the new software with FFPE samples and other solid sample types. METHODS: Four unique FFPE tissue samples were purified using the fully-automated IVD DNA protocol and the new DNAext protocol. DNA eluate yields from three sample replicates were compared between both extraction protocols. In addition, four replicates each of fresh frozen (FF) bone marrow tissue*, cultured cell lines*, and peripheral blood mononuclear cells (PBMC)* were extracted with the Tissue Preparation Solution. Paired DNA and RNA eluates (n = 4) from each sample were generated using the IVD SPLIT protocol. Nucleic acids extracted using the TPS protocols were qualified with in-house DNA and RNA surrogate gene real-time PCR/RT-PCR assays. The DNA and RNA eluates were also qualified using the industry-standard QUANT-IT PICOGREEN dsDNA and QUANT-IT RIBOGREEN RNA ASSAY KIT. RESULTS: When comparing the two DNA extraction protocols, the DNAext protocol, with increased lysis conditions, showed enhanced recovery of DNA from FFPE tissue samples. Furthermore, the SPLIT protocol extracted both DNA and RNA from a single sample of FFPE tissue, bone marrow, cell cultures, and PBMC. The extracted nucleic acids met all in-house acceptance criteria, demonstrating being both amplifiable and free of any inhibitory contaminants. In addition, commercially available qualification assays demonstrated that the DNA yield and integrity are suitable for molecular applications such as PCR and sequencing. CONCLUSION: The Tissue Preparation Solution is capable of extracting high-quality DNA and RNA from FFPE tissue, FF tissue, bone marrow, cell cultures, and PBMC. The new TPS software with the DNAext protocol successfully extracted FFPE tissue DNA at higher concentrations than the DNA protocol. The Tissue Preparation Solution offers flexibility for processing multiple solid sample types using one instrument and universal IVD reagents. The newly developed Tissue Preparation Software version 2.1 provides workflow advantages by generating DNA and/or RNA eluates within a single extraction.

*Not a validated sample type.

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Multiplex Ligation-Dependent Probe Amplification (MLPA) as a diagnostic tool for detection of Williams-Beuren syndrome (WBS).

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Williams-Beuren syndrome (WBS) is caused by a hemizygous contiguous gene microdeletion of the critical region on chromosome 7 at a position 7q11.23, which contains approximately 28 genes. Patients with WBS have specific dysmorphic features and are characterized by growth deficiency, mild cognitive delay with relative strength in expressive language, overfriendliness, hypercalcaemia and a supravalvular aortic stenosis (SVAS). The WBS is estimated to occur at a frequency of approximately 1 in 7500 live births with no ethnic or sex bias. Familial cases have been reported with apparent autosomal dominant inheritance. The most common deletion including the ELN gene is found in approximately 90-95% of the clinically typical WBS patients but in a lower percentage of atypical cases. The commonly deleted or duplicated chromosomal region has a size of approximately 1.5 to 1.8 Mb and is flanked by two highly homologous DNA sequences. However, smaller deletions involving only the ELN gene or the ELN and LIMK1 genes have also been described in SVAS and atypical WBS patients. Multiplex Ligation-Dependent Probe Amplification (MLPA, MRC-Holland) has been introduced into DNA diagnostic laboratories for the detection of molecular genetic alterations that are of diagnostic and prognostic significance. The MLPA kit for WBS, when compared to other techniques, is capable of detecting smaller and atypical deletions and duplications in the WBS critical region. Twenty patients with diagnosis hypothesis of WBS were tested using a commercial MLPA kit P029 specific for WBS and thirteen with kit P064 designed for mental retardation. The analysis was performed using the GeneMarker v2.6.2 software. Furthermore, 7 patients tested with Kit P029, were also evaluated by Short Tandem Repeat technique (STR) using 3 markers (HEI135, LINK1, D7S5613). Results obtained with MLPA kit P029 were concordant with MLPA kit P064 in all cases tested (n=13). The microdeletion was present in 3 patients and absent in 10. Using the STR technique, the results were concordant with MLPA kit P029 in 4 patients. However, we found 3 discrepant results comparing the microsatellite markes and MLPA method. The STR technique was not informative in these 3 cases since the absence of amplification of one allele does not discriminate the hemizygous from the homozygous condition that often leads to diagnostic difficulties the WBS. The most important advantages of the MLPA are its relative simplicity, low cost, rapid turnaround (2 days), ease of multiplexing to permit high confidence in the results, high accuracy of copy number estimation, and the potential for combination of copy number analysis with other applications. In conclusion, MLPA consists in a highly informative and easily manageable tool for the diagnosis confirmation of WBS and a faster and more economical method carried out in a single assay for the detection of deletions in WBS.

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Detection of microdeletion syndromes by MLPA: A case of validation

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BACKGROUND: Chromosomal rearrangements that result in deletions and duplications of the part of the chromosome are among the main causes of congenital abnormalities. About 15-25% of the changes detected are microdeletions or microduplications, such as Velocardiofacial (del 22q11.2), Angelman and Prader-Willi (del 15q11) and Wolf-Hirschhorn(del 4p16) syndromes. The remaining cases are caused by other subtelomeric changes, balanced translocations, inversions, insertions, or mosaicism. Alteration of gene dosage due to gains or deletions of large genomic regions causes many genetic disorders that are frequently associated with intellectual disability (ID),multiple congenital anomalies (MCA), autistic spectrum disorders (ASD) and other phenotypic findings. Intellectual disability is characterized as impaired cognitive function and deficits in two or more adaptive behaviors and affects 1-3% of all children and cause a great impact on the lives of patients and their families. Understanding of the ID etiology is essential for guidance and genetic counseling for families as well as for the establishment of preventive measures.The Resolution of conventional karyotype isn't appropriate to detect small (less than 5-10Mb) genetic alterations under the microscope. However, techniques of molecular cytogenetics, having higher resolution, such as FISH (Fluorescent in situ Hybridization), MLPA (ligation-dependent probe amplification multiplex) and genomic microarray screening reach double the detection rate of chromosomal abnormalities.Our work aimed to verify the performance of the MLPA P064 kit in the laboratory's routine by comparing the results obtained in our verification and the results from 22 samples studied by MLPA at the Institute of Education and Research of Santa Casa Belo Horizonte - IEPSCBH.

METHODS: The MLPA p064 reaction was carried out following the manufacturer's instructions with little modifications. The Kappa statistic was used to calculate the concordance between the tests.

RESULTS: Among 22 samples, 2 individuals were positive to two different syndromes. A third individual had a positive result for one of the syndromes detected by the kit but he doesn't was considered a discordance because the different versions of kit used to do the test. Result's accordance obtained from these 22 samples using

Kappa statistics was perfect 1,0 (0.582 to 1.0 CI 95%). There was no statistically relevant difference ($p < 0.001$) among compared results.

CONCLUSIONS: We concluded that the kit MLPA P064 could be used in the laboratory's routine for multiplemicrodeletionsyndromes simultaneously.

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CX3CR1 mRNA expression is related to weight gain in obese individuals from Brazil

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Background: Obesity is cosmopolitan endemic disease caused by an imbalance between energy intake and expenditure. Pro-inflammatory status has been suggested to be involved in the adipogenesis, and similar to atherogenesis, may increase the susceptibility to cardiovascular disease. Polymorphisms in genes encoding proteins involved in the inflammatory process may contribute to the risk for obesity. Objective: To investigate whether polymorphisms e mRNA expression in inflammatory-related genes are associated with obesity in a Brazilian population. Methods: One-hundred-ninety-nine individuals attended at two Medical Centers of the Sao Paulo city, Brazil. were enrolled in this study. Anthropometrics, fat mass and clinical variables were recorded and blood samples were taken for DNA and RNA extraction and analysis of metabolic and inflammatory markers and adipokines. Individuals were grouped as lean (n=40, BMI: 18.5-24.9 kg/m2), overweight (n=55, BMI: 25.0-29.9 kg/m2) and obese (n=104, BMI: > 30kg/m2). CD40 (rs1883832) C>T, CX3CR1 (rs3732379) C>T, CX3CR1 (rs3732378) G>A, E-selectina (rs5368) C>T, ICAM-1 (rs1799969) G>A, ICAM-1 (rs281432) C>G, LIGHT (rs344560) G>A, LIGHT (rs2291668) C>T, RAGE (rs2070600) G>A, RAGE (rs2236493) C>T e VCAM (rs3176878) C>T gene polymorphisms and mRNA expression in peripheral blood leukocytes were analyzed by pyrosequencing and qRT-PCR, respectively. PAI-1, IL-6, TNF-α, resistin, adiponectin, and leptin were determined by and Luminex xMAP technology, and CRP-hs by imuno-nephelometry. Results: Serum CRP-hs, PAI-1, IL-6 and TNF-α were higher in obese than in lean group ($p<0.001$). The studied polymorphisms were not associated with obesity, and anthropometric and laboratory variables. Univariate logistic regression analysis showed that weight gain (increased BMI) was directly related to CX3CR1 mRNA expression (OR: 4.24, 95%CI: 1.27-14.08, p=0.018). Conclusions: These results are suggestive that mRNA expression of CX3CR1 increases with weight gain, reflecting the inflammatory status of obese individuals and, therefore, may be a useful biomarker.

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Frequency of thrombophilic mutations associated with karyotype in women in laboratory investigation for infertility.

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Introduction: Infertility female factors can occur in 5 to 25% of cases, male causes 30 to 40% of cases and 10 to 15% cause can not be determined. Furthermore, infertility can be influenced a woman's age, frequency of sexual intercourse and body weight, therefore different diagnostic and therapeutic approaches may apply. The failure of embryo implantation is considered a relevant cause of failure in *in* vitro fertilization procedures. The blood disorders leading to hypercoagulability, in other words, thrombophilia may compromise the process of embryo implantation. Therefore, the interest in improving the embryo implantation rates led to the study of angiogenesis at the implantation site and, consequently of diseases related to alterations in blood coagulation system. The relationship between the thrombophilic factors and infertility should be considered as a possibile cause of spontaneous early miscarriage, caused by alteration in hemostasis of thrombophilic origin, at the implantation site. This abnormality affects vascular Trophoblastic invasion and placental vasculature.

Different genetic factors may contribute to infertility, but chromosomes number and structure stabilty is the main one. It is estimated that approximately 0,65%, i.e. one out of 153 live births, have chromosomal abnormalities, in cases of miscarriage, this number reaches reaches approximately 48,8%.

Chromosomal heteromorphisms are structural variants common in the population, without apparent phenotypic effect. It has been reported the increased incidence of chromosomal heteromorphisms in cases of infertility, recurrent miscarriages and other changes.

Method: We aimed to evaluate the prevalence of factors of hereditary and acquired thrombophilia, and its associaton with karyotype abnormalities, in females reffered to a large reference laboratory. We retrospectively surveyed LIS database, from January to December 2014, for concomitant results of thrombophilic alterations (Factor V Leiden; Antithrombin III; gene mutations on C677T, A1298C and MTHFR, and in prothrombin gene) and G-band Karyotype in females aged between 18 and 50 years-old.

Result: Eighty six women fitted the inclusion criteria, 59% [51/86] presented some type of alteration on coagulation related factors studied. Twenty seven (53%) of those with thrombophilic abnormalities presented some chromosomal heteromorphisms, found in chromosomes 1, 9, 15 and 21. And also, one woman (1.96%) presented translocation on chromosome 46,XX,t(11;17)(p11.2;p13), associated with alteration of antithrombin.

Conclusion: The heteromorphisms have been observed with increased frequency in infertile couples, with history of miscarriages, parents of children with chromosomal abnormalities, and chromosomally abnormal live births. The inefficient placental blood flow caused by thrombophilic events or vascular insufficiency can lead to deleterious effects on the development of pregnancy. Therefore the association chromosomal and thrombophilia or both disorders may contribute aditionally to female infertility. This study showed that more than half of women reffered to a large laboratory for infertility investigation presented both conditions together. This association may increase the severity of infertility and, therefore, it is recommended to evaluate Karyotype and thrombophilia, in order to better understand the causes and manage female infertility.

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Screening of alterations in copy number variation (CNV) in subtelomeric regions by MLPA as test diagnosis for patients with congenital malformation and disability intellectual

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Alterations in copy number variation (CNV) represent about 3-8% of potential cause of delay development, dysmorphic featuresand congenital abnormalities in subtelomeric regions. In these regions there are genes flanked by short repetitive sequences of nucleotides that are highly unstable. The detection of abnormal copy numbers in subtelomeric regions should be done carefully because the subtelomeric copy number changes can also occur in unaffected individuals and the effect of a deletion or duplication will depend on the genes involved. The detection of abnormal copy numbers in subtelomeric regions should be done carefully because the subtelomeric copy number changes can also occur in unaffected individuals and the effect of a deletion or duplication will depend on the genes involved. Nowadays, there are many methods to study deletions and duplications in the whole genome as fluorescent in situ hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA) and whole-genome array screening. The study of deletion and duplications in subtelomeric regions by MLPA technique is an alternative more accessible and suitable for quantification of CNV. The aim of this study was to validate two P036 and P070 MLPAkits to investigate CNV in subtelomeric regions of the 23 pairs of chromosomesin a clinical laboratory.

The samples of 31patients of both sexes, with age birth to adulthood were collected at Institute of Education and Research of Santa Casa de Belo Horizonte - IEPSCBH and tested with the kit P036 and P070 Human Telomere in parallel with Laboratory of Human Genetics - Institute Hermes Pardini. The MLPA reaction was carried out following manufacturer's instructions. GeneMarker® software was used to analyze the electropherograms. The Kappa statistic was used to compare the results.

Our results were concordant in 100% of analyzes.The kit P036 found three alterations, in the chromosomes 4p16.3, 8p23.3 and 22q 11.21 (9.67%) and the kit P070 found two alterations, in the chromosomes 4p16.3 and 8p23.3 (6.45%) in the samples of 31 patients.The difference in detection of chromossome 22 is because the region investigated by P070 is other (22q13.33), so the use of both kits concomitantly can improve detection rate of alterations.

We concluded that the kit MLPA P036 and P070 could be used in the laboratory's routine and we recommended the use of both concomitantly to improved detection rate of the alterations.