Wednesday, July 30, 2014

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

Molecular Pathology/Probes

B-185

Case Report: Molecular and Cytogenetic characterization of a 46,XX male

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Background: The Y chromosome evolves from an auto chromosome and accumulates male-related genes including sex-determining region of Y-chromosome (SRY) and several spermatogenesis-related genes. 46,XX subjects carrying the determining SRY gene usually have a completely male phenotype.

Objective: To identify Y chromosome material in an azoospermic male with a 46, XX karyotype.

Method: A 39 years old male was tested to micro deletions of Y chromosome in order to investigate the origin of an infertility characterized by azoospermia. He did not report any previous familiar history of infertility and was never submitted to any infertility treatment before. The isolated DNA was submitted to a PCR reaction to amplify the following regions of Y chromosome according to the protocol previous described by Simoni et al (2004). The patient was also submitted to a karyotype analysis which shown presence of two X chromosomes. To confirm the presence of Y or part of chromosome, fluorescence in situ hybridization (FISH) was performed using LSI SRY/CEP X probe set (Vysis®).

Results: PCR amplification of DNA was detected using QIAxcel DNA Screening Kit (Qiagen, Hilden Germany) and showed the presence only of the sex-determining region of the Y chromosome (SRY) and the absence of others target regions of Y chromosome (AZFa, AZFb and AZFc). FISH analysis showed an X chromosome containing SRY gene sequence on the top of the short arm. This Y chromosome gene was not visible by conventional cytogenetic analysis which shown presence of two X chromosomes.

Conclusion: Molecular and FISH techniques were very useful for detecting and locating Y sequences in this particular case, allowing an accurate diagnosis and correct management of the patient. Testing new Y chromosome markers in XX males will make it possible to narrow the breakpoints further in each case and to establish correlations with the clinical features, identifying the Y regions implicated in the definition of the phenotype.

B-188

Validation of molecular testing of the Y chromosome microdeletions in DNA analyser: A case of laboratory automation

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BACKGROUND: Infertility is a medical condition that affects 10-15 % of couples seeking to have children.Large proportion of cases of idiopathic male infertility is due to the presence of microdeletions in the Y chromosome genes related to spermatogenesis. Located on the long arm of the chromosome Y, the 3 regions known as “azoospermia factors” (AZFa , AZFb and AZFc) are found fully or partially deleted in azoospermic patients or patients with severe oligozoospermia. Our laboratory offers the test for Y chromosome microdeletions by multiplex PCR followed by electrophoresis on 2% agarose gel stained with ethidium bromide. Once it is a manual method occasional errors may occur in prepare the gel or pipetting the samples. Furthermore, ethidium bromide is a mutagenic reagent and therefore the represents a risk to both the operator and the environment.

OBJECTIVE: The objective of this study is to standardize the diagnosis of Y chromosome microdeletions through a more efficient and secure methodology.

METHODS: For this study, the capillary electrophoresis was chosen to substitute the agarosegel electrophoresis. Modifications were made in the PCR reactions, the primers were labeled with different dyes and the settings in the analysis software GeneMapper ID-X v. 1.1.1 were modified to optimize theanalysis of this genetic testing in different fluorescence channels in the 3730 DNA Analyzer.In order to evaluate such changes, the results of 35 patients who carried out themicrodeletions of the Y chromosome were both analyzed on agarose gel and incapillary electrophoresis. The Kappa statistic was used to compare the results.

RESULTS: Results accordance obtained between the two techniques fromthe 35 samples using Kappa statistics was perfect 1,0 (0,669 to 1,0 CI 95%). There were no statistically relevant difference ($p < 0,001$) among compared methodologies.

CONCLUSIONS:We concluded that capillary electrophoresis could be used as an alternative method for the study of Y chromosome microdeletions, providing a more efficient and secure assay.

B-189

Integrative analyses of Hippo pathway components in human cancer genome

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Background: The Hippo signaling pathway regulates cell proliferation and apoptosis to control tumor growth and organ size. Cancer genome projects found more somatic mutations in Hippo pathway than candidate approach. However, it is elusive for their damaging degree and their association with other mutated genes in the signal transduction network. In here we have analyzed the somatic non-synonymous mutations of the Hippo pathway components from cancer genome database.

Methods: Hippo pathway components mutation data are downloaded from COSMIC (Catalogue Of Somatic Mutations In Cancer) and TCGA (The Cancer Genome Atlas). The conservation of the mutated residues and the 3D structure remodeling are evaluated by ClustalW2 and CPH models 3.2. All the mutation pathogenicity are determined by PolyPhen2, Mutation Assessor, SIFT and Provean. We also integrated knowledge of the mutated sites from literature using candidate mutagenesis, or large scale phosphoproteomics approaches. The carcinoma genome of ten tissues with top mutated frequencies of Hippo Pathway components are downloaded from TCGA data portal. Significant concurrent and mutually-exclusive relationships ($p\text{-value}<0.01$) were revealed among the mutations of the Hippo components, its interactome, and cancer census genes. The unbiased screening selected a list of genes with strong associations with Hippo components. The significantly mutated genes were identified from 57, 57, 28 and 25 patients carried predicted damaging mutations of LATS1, LATS2, STK3 and STK4, and compared with the ones found in 19, 33, 32, and 27 patients with synonymous or predicted neutral mutations. Survival analyses were also performed for the patients with Hippo pathway alterations.

Results: The mutation spectrum of Hippo Pathway components has been explored. Core Tumor suppressors(LATS1/2 and STK3/4) (5%) have higher damaging mutation frequency than core oncogenes (YAP1/WWTR1 and TEAD2/4) (1%) in the Hippo pathway. 67% out of total 652 mutations and 36 predicted damaging mutations sit in the domain and residues, respectively, reported by literature. Hippo interactome test found that PSMD2, WWP1, NEDD4 involved in the degradation of tumor suppressors in Hippo pathway are found concurrent mutated with those tumor suppressors. Cancer census gene comparison testing found that XPO1, TCEA1 and SUZ12 are concurrent with Hippo pathway components, while PIK3CA, TP53 and STK11 are mutually exclusive. The unbiased screening identified CENPC1, ORC are concurrent with Hippo pathway components, while CDKN2A and RHOA are mututully exclusive. Patients with up-regulation of oncogenes has worse survival rate than the patients without.

Conclusions: The analyses for Hippo Pathway mutations in cancer patients provided comprehensive reference for physicians and researchers to further investigate the mutations. The genomic analyses shed light on elucidating mechanisms of Hippo pathway and the concurrence and mutually exclusivity with its associated genes in cancer, which could aid in the development of a cancer biomarker panels for prognoses and treatments.

B-191

Comparative Genomic Hybridization arrays (aCGH) Technique as a diagnostic tool for 22q11.2 microduplication syndrome.

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Background: At the moment, it`s uncertain whether the 22q11.2 microduplication is a natural genetic variant-we are all different-or whether it`s a real syndrome whose effects can be highly variable. The 22q11.2 syndrome is related to development delay, intellectual disability, slow growth leading to short stature and poor muscle tone. All patients underwent examinations of karyotype and in all cases were normal. Most people have a 22q11.2 microduplication that is about <3 Mb in size. Most of the 30-40

genes in the <3Mb stretch of duplication have not been fully characterized. However, researchers believe that the duplication of one particular gene, known as *TBX1*, is responsible for many of the typical symptoms of the syndrome. aCGH is a molecular cytogentics technique, able to identify unbalanced chromosomal changes (gains or losses of genomic material) through the general analysis of the entire genome in a single experiment. Objective: The objective of this study was to show the use of the technique Comparative Genomic Hybridization arrays (aCGH) as a molecular tool for the diagnosis of microduplication 22q11.2. Methods: 3 samples of peripheral blood of pediatric patients referred to the Institute for study of loss or gain of genomic material were used. The samples were collected in tubes 4ml EDTA and stored at 4 ° C until processed. For DNA extraction was used *QiampDNA Blood Mini Kit* (*Qiagen*). After extraction the samples were quantified and evaluated reasons for A260/A280 and A260/A230 in NanoDrop2000/2000c. The samples had concentrations above 50 ng/uL and A260/A280 (1.8 to 1.9) and A260/A230 (1.5 to 1.9) were among the reasons for standard values. gDNA was digested with restriction enzyme labeled with the cyanines 3 (Cy3-reference) and 5 (Cy5-patient) and purified by *SureTag Complete DNA Labeling Kit* (*Agilen*t). After purification, the samples were again quantified to measure the incorporation of Cyanine 5 (Cy5). All samples had A260/A280 reason to 1.8, DNA concentration above 420.0 ng/uL, the incorporation of Cy5 was above 10.3 pmol/uL, the specific activity was above 24 pmol Cy5/µg DNA and yield ug DNA was above 8.9. The *OligoaCGH/ChIP-on-chip Hybridization Kit* (*Agilent*) and *Human Cot-1 DNA* (*Agilent*) kit was used to perform hybridization on the slide containing probes corresponding to 180,000 genes. After 24 hours of hybridization at 65 ° C the slides were washed with *AgilentOligoaCGH/ChIP-on-Chip 1* and *2 Wash Buffer Kit* (*Agilent*) and acetonitrile reagent (*Sigma*). After extraction of data through the Software *ScanControle Software Fecture Extraction*, these were analyzed in *Agilent CytoGenomics* Edition Software 2.7.8.0. Results: All samples met the standards of quality required for the analysis. The technical benchmark *DerivativeLR_Spread* (DLRS) was <0.30. Samples showed gain of genomic material (279 Kb) in the 22q11.2 region. Conclusions: This study shows the importance in examining the aCGH technique for the diagnosis of patients with 22q11.2 syndrome who had normal results in the karyotype.

B-192

Genotyping of rs12979860 and rs8099917 single nucleotide polymorphisms in HCV infected Brazilian patients.

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Background: Recent studies have demonstrated the role of the interleukin 28B (IL28B) polymorphisms in predicting treatment response, spontaneous clearance and sustained virologic response for patients with Hepatitis C virus (HCV) infection. Two main single nucleotide polymorphism (SNP) were identified in proximity to interleukin IL28B: rs12979860 (homozygous for the major C allele are more likely to respond to treatment than those who were homozygous for the alternative nucleotide (T) and rs8099917 (strong predictor of sustained virologic response (SVR) to pegylated interferon and ribavirin for subjects with homozygous T/T genotype than subjects with G/G genotype).

Objective: To describe the frequency of the IL28B C/T SNP for rs12979860 and that of the T/G SNP for rs8099917 in a cohort of Brazilian patients with HCV infection.

Methods: Blood samples from 305 HCV infected Brazilian patients were analyzed from January 2013 to December 2013. DNA isolation was performed with the automated platform Qiasymphony SP (Qiagen, Hilden, Germany) according manufactured instructions. Genotyping for rs12979860 and rs8099917 were performed using TaqMan® SNP Genotyping Assays (Life Technologies, Foster City, CA) at Viia 7 Real Time PCR System (Life Tecnhologies). The results were analyzed using TaqMan® Genotyper Software version 1.0.1.

Results: Carriers of rs12979860 CT genotype predominated (160/305, 52.5%), homozygotes for allele C were 90/305 (29.5%) and the remaining homozygotes for IFN-resistant allele T were 55/305 (18.0%). As for the rs8099917 SNP, genotypes were distributed as follow: 175/305 (57.4%) carried the rs8099917 TT genotype, whereas 110/305 (36.0%) carried GT and 20/305 (6.6%) the GG genotype. The co-prevalence of genotypes is shown at Table 1.

Conclusion: The frequencies found were consistent with previous studies. Testing for rs12979860 and rs8099917 has become an important strategy to predict the patient treatment outcome.

Table 1- Combined genotype frequencies of the interleukin (IL)28B single nucleotide polymorphisms rs1297860 and rs8099917

	rs8099917			
	GT	**GG**	**TT**	**Total**
rs12979860	n (%)	n (%)	n (%)	
CT	81 (26.6)	2 (0.6)	77 (25.3)	160 (52.5)
CC	4 (1.3)	0 (0.0)	86 (28.2)	90 (29.5)
TT	25 (8.2)	18 (5.9)	12 (3.9)	55 (18.0)
Total	110 (36.0)	20 (6.6)	175 (57.4)	305 (100)

B-193

Validation of Hybridizing Genomic Comparative array (aCGH) technique to screen 180.000 genes in diseases Postnatal.

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Background: aCGH technique has been widely used in postnatal diagnosis of patients with normal karyotyping and disturbs like autism, neuropsychomotordevelopmental delay, facial dimorphism, mentaldevelopment delay, low weight and low height, among others. Because of its capacity to detect microdeletions and microduplications, aCGH is an important tool to investigate such clinical cases. Objective: The objective of study was validation and implements the Hybridization Genomic Comparative array (aCGH) technique for postnatal diagnosis of normal karyotyping patients. Methods: Samples from 12 patients were analyzed in duplicate by Hermes Pardini Institute and a reference laboratory. The samples were collected in tubes 4ml EDTA and stored at 4 ° C until processed. For DNA extraction was used *QiampDNA Blood Mini Kit* (*Qiagen*). After extraction the samples were quantified and evaluated reasons for A260/A280 and A260/A230 in NanoDrop2000/2000c. The samples had concentrations above 50 ng/uL and A260/A280 (1.8 to 1.9) and A260/A230 (1.5 to 1.9) were among the reasons for standard values. gDNA was digested with restriction enzyme labeled with the cyanines 3 (Cy3-reference) and 5 (Cy5-patient) and purified by *SureTag Complete DNA Labeling Kit* (*Agilen*t). After purification, the samples were again quantified to measure the incorporation of Cyanine 5 (Cy5). All samples had A260/A280 reason to 1.8, DNA concentration above 420.0 ng/uL, the incorporation of Cy5 was above 10.3 pmol/uL, the specific activity was above 24 pmol Cy5/µg DNA and yield ug DNA was above 8.9. The *OligoaCGH/ChIP-on-chip Hybridization Kit* (*Agilent*) and *Human Cot-1 DNA* (*Agilent*) kit was used to perform hybridization on the slide containing probes corresponding to 180,000 genes. After 24 hours of hybridization at 65 ° C the slides were washed with *AgilentOligoaCGH/ChIP-on-Chip 1* and *2 Wash Buffer Kit* (*Agilent*) and acetonitrile reagent (*Sigma*). After extraction of data through the Software *ScanControle Software Fecture Extraction*, these were analyzed in *Agilent CytoGenomics* Edition Software 2.7.8.0. Results: All samples met the standards of quality required for the analysis. The technical benchmark *DerivativeLR_Spread* (DLRS) was <0.30. Results for the same patient were compared between both laboratories and all of them had the same sensibility, specificity and reproducibility. Four patients had at least one pathogenic alteration detected by aCGH. One had many LOH regions detected showing that this patient's parents came from the same ancestor. The other seven had alterations considered non-pathogenic. Pathogenic alterations detected were a deletion on the chromosome 4 short arm (4p16.3p16.2), that overlaps the Wolf-Hirschorn syndrome region; a duplication on the chromosome 9 short arm (9p24.3p24.2), related to developmental delay; a deletion on the chromosome X long arm (Xq27.2), related with mental development delay and obesity; a duplication on the chromosome 7 long arm (7q36.3), also related with mental development delay; and a deletion on the chromosome 15 long arm (15q11.2-q13.1), that overlaps Prader-Willi and Angelman syndromes region. Conclusions: Concluding, almost half of the patients presented a pathogenic alteration that was not detected by conventional karyotyping proving the importance of aCGH as a complementary diagnosis tool.

B-194

Comparison of Invivoscribe's T Cell Receptor Gamma Gene Rearrangement Assay 2.0 vs TCRG Gene Clonality Assay (developed by the Euroclonality, previously BIOMED-2 Group)

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Background Histology or cytology, supplemented with immunohistology or flow cytometric immunophenotyping has been used to discriminate between malignant and reactive lymphoproliferations. However, in some of the cases, the diagnosis is difficult. The diagnosis of lymphoid malignancies can be supported by clonality assessment as all cells of a malignancy have a common clonal origin. Gene rearrangement analysis used to be performed by Southern Blot-based techniques which is very reliable, but is increasingly replaced by PCR techniques because of

the greater efficiency and sensitivity of PCR. These gene rearrangements generate products that are unique in length and sequence for each cell. Therefore, PCR assays can be used to identify lymphocyte populations derived from a single cell by detecting the unique V-J gene rearrangements present within these antigen receptor loci. We evaluated the T cell receptor gamma gene clonality assay (Euroclonality's primer) and T cell receptor gamma gene rearrangement assay 2.0, both from Invivoscribe, for clonality assessment of T cells lymphoproliferative disorders.

Methods 22 archived formalin-fixed, paraffin-embedded (FFPE) clinical samples were extracted with QIAamp DNA FFPE tissue kit, following manufacturer's recommendation. 2 proficiency panel samples were included as well. The extracted genomic DNA was quantified and amplified using the specimen control size ladder master mix in the Invivoscribe kits and AmpliTaq Gold DNA polymerase. All samples were tested in duplicates of 50ng and 100ng of DNA to check for presence of inhibitors and DNA quality. Next, PCR was performed with TCRG tube A, TCRG tube B and TCRG 2.0 with samples in duplicate, polyclonal, monoclonal and negative control. The PCR products were denatured with Hi-Di formamide and GeneScan 600 Liz size standards and anlayzed on ABI 3500 capillary electrophoresis instrument. Data are automatically displayed as size and color specific peaks.

Results Majority of the samples were concordant with the in-house developed test for TCRG, TCRG clonality assay and TCRG 2.0. However, 2 of the samples showed discrepant results. TCRG 2.0 requires shorter hands-on time to perform and is easier to analyze as it is single tube and single color (blue). TCRG clonality assay has 2 tubes (tube A and B) and each tube is dual-color (green and blue). The dual-color can be quite confusing to interpret. The clone size detected is different for both assays as the PCR primers are different. Hence, for different labs using these 2 assays, it is hard to correlate if the clone detected is the same.

Conclusion The TCRG clonality assay and TCRG gene rearrangement assay 2.0 from Invivoscribe generated similar results for clonality assessment of T cells lymphoproliferative disorders. We had 2 cases with discrepant results and will emphasize that the results of molecular clonality tests must always be interpreted in the context of clinical, histological and immunophenotypic data. TCRG clonality assay adopts the Euroclonality's primers and is more widely used in laboratories. Even though TCRG gene rearrangement assay 2.0 is easier to perform and interpret, more data should be generated for the better comparison of these 2 assays.

B-195

HPV E6/E7 mRNA RT-qPCR Assay for Detecting High Grade of Cervical Lesion with ThinPrep Pap Samples

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Background: Human cervical cancer is the second most common cancer among women worldwide. Several decades ago, human papillomaviruses (HPV) were found out to be a major factor of cervical cancer. HPV DNA genotyping assay has been the method of the choice, since it has shown high analytical sensitivity. The latest results show oncogenic HPV DNA appeared not only in cancerous tissues, but also in the normal tissues according to cytological diagnosis. For this reason, HPV test targeting E6 and E7 mRNA of 5 oncogenic HPVs (HPV genotype 16, 18, 31, 33, and 45) which are known to be responsible for oncogenesis of cervical cancer has been commercialized using real-time nucleic acid sequence based amplification (NASBA) assay. The previous data showed that the real-time NASBA assay has higher clinical specificity than HPV DNA testing (97.1 % vs. 53.7 %). However, the sensitivity of real-time NASBA assay was lower than that of the HPV DNA testing (41.1 % vs. 100 %).

Methods: In the present study, therefore, HPV E6/E7 mRNA targeting RT-qPCR assay was designed to detect 16 oncogenic HPV genotypes (HPV genotype 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 69), it was performed with RNA prepared from ThinPrep® Pap (Hologic Inc., Bedford, MA, USA) samples, and the results were compared to real-time NASBA data.

Results: For the detection of $CIN2^+$ high grade cervical lesions, the sensitivity and specificity of RT-qPCR assay were 92 % and 98.6 %, respectively. Therefore, HPV E6/E7 mRNA RT-qPCR assay showed the significantly higher sensitivity (91.1 %) compared to real-time NASBA assay (41.1 %). In normal cytology cases, the specificity was 98.6 % and 53.7 % by HPV E6/E7 mRNA RT-qPCR assay and HPV DNA testing. These results revealed that HPV E6/E7 mRNA RT-qPCR assay better reflects cytological diagnosis.

Conclusion: It is suggested that Real-HPV E6/7 mRNA® assay (M&D, Wonju, Republic of Korea) could overcome the shortcoming of lower specificity in DNA assay as well as the lower sensitivity of commercialized HPV mRNA real-time NASBA assay, NucliSENS EasyQ HPV v1.1 (bioMérieux, Marcy, France), with ThinPrep® Pap (Hologic Inc.) samples.

B-196

Donated organ genomic signature in circulating DNA of the liver transplant recipient to monitorization the transplanted liver health

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Background:Health assessment of the transplanted organ is very important due to the relation of long-term survival of organ transplant recipient and the maintaining of organ health. It has been described that transplanted organ cells suffering damage liberate DNA. Thus, during organ rejection, apoptosis cell death deals to the release of specific transplanted organ DNA to the host plasma. In this context organ cell free DNA may give us a differential genomic marker of the donated organ health. In a first approximation the quantification of DNA from Chromosome Y in women host under male organ transplantation may be a useful tool. The objective of this work was to validate the usefulness of quantifying specific organ circulating DNA (cDNA) in serum of transplanted patients as noninvasive diagnostic genomic marker, in the diagnosis of graft injury. With this purpose we monitorized serum cDNA from chromosome Y in eight women after male liver transplantation.

Methods: cDNA quantization of the SRY gene was performed by real-time quantitative PCR before, at the moment of transplantation (day 0) and during the stay at the intensive care unit. Beta-globine cDNA levels, a general cellular damage marker, were also quantified. Patients were grouped based on clinical outcome. Group A, were patients that accepted liver transplantation without any complication (Patients 1-3); group B were patients that accepted the male organ but suffer complication not related with the transplantation (patients 4-6); patient 7 suffering an autoimmune hepatitis rejected the first transplanted organ but accepted the second one, and finally patient 8 underwent a liver transplantation from a male liver donor suffering a sepsis by colangitis that developed to general organ failure and died.

Results: All patients showed an increase of cDNA levels at the moment of transplantation that decreased until patient stabilization. Group A, showed the early peak at day 0 that immediately disappear. Patients from group B showed an increase of beta-globine gene levels but not of SRY gen ones at the moment of any clinical complication. Patient 7 showed high levels of of beta-globine gene levels and SRY gen after the first transplantation which was rejected and decreased after the second one accepted. Liver transplantation was succeful for patient 8 showing low levels of SRY gen during most the first weeks after surgery. However the beta-globine levels persisted elevated due to a colangitis that ended in a sepsis, multiorganic failure and death. At the moment of multiorganic failure SRY gen levels were also increased.

Conclusion: Our results shows that donor-derived cDNA may be quantified in the serum of organ transplant recipients, and that high levels of donor DNA might be used as an indication of graft injury vs other pathologies of the patients

B-197

Genetic variants of glucose-6-phosphate dehydrogenase (G6PD) in Brazilian children with positive neonatal screening for G6PD deficiency, and correlation with neonatal jaundice

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Background: G6PD deficiency, the most common human enzymopathies throughout the world, causes a spectrum of phenotypes including neonatal hyperbilirubinemia and acute and chronic hemolysis. Genetically, G6PD deficiency is a heterogeneous

condition, with approximately 150 mutations and 400 variants identified. Molecular studies seek to define the origin of the enzymopathy in a determined population and correlate the G6PD variants with the clinical course of the disease, as well as identifying the G6PD deficiency in heterozygous females. Previous reports have shown that the prevalence of G6PD deficiency, in several regions of Brazil, is around 10% among males of African origin and between 1-6% on euro-descendent males. The data regarding the types of G6PD variants in Brazilian population are fragmented and scarce. The capital of Brazil, which lies in the Federal District, has a mixed population representing the different regions of Brazil. The Neonatal Screening Program (NSP) in Federal District indicates a prevalence of 4.5% for G6PD deficiency. The objective of the current study was identify the types of variants in the G6PD gene in a group of children screened through the NSP in the Federal District, and correlate these data with the presence of neonatal jaundice. Methods: Oral mucosa samples were collected from eighty boys and four girls diagnosed with G6PD deficiency through the NSP in January and February of 2014, whose parents signed an informed consent form. The majority of the newborns presented with residual enzyme activity of around 50% (moderate deficiency). All representatives of the children filled out a questionnaire with relevant details regarding family history, history of neonatal jaundice and therapy. Molecular analysis was carried out using real-time PCR (allelic discrimination). The G202A and C563T mutations in the G6PD gene were analyzed using specific primers and probes. Results: Seventy of the 84 families were unable to provide information regarding ethnic origin of the child, 13 claimed indigenous descent, and one claimed Portuguese and Spanish descent. 60.7% of the children presented with neonatal jaundice, 76.5% presented at 48 hours post-natal, and 29% required phototherapy. Molecular analysis identified a high proportion (98.8%) of neonates positive for the G202A mutation (variant G6PD A-): 79 boys were hemizygous and 4 girls were homozygous for this mutation. Only one boy presented the Mediterranean C563T mutation. Analysis of the correlation between genotype and presence of neonatal jaundice was compromised by the intense predominance of the G202A mutation in the sample group. Conclusions: This is the first study carried out in the population of individuals with G6PD deficiency in the Federal District of Brazil. Although the sample group studied was relatively small, the high prevalence of a single mutation suggests that G6PD deficiency in the population of the Federal District is principally due to the G202A mutation. Neonatal jaundice was frequent among G6PD deficient children. The absence of cases of heterozygous females in the sample group may reflect the inability of neonatal enzyme screening to detect G6PD deficiency in these cases.

B-199

Validation of a quantitative CMV Simplexa kit for clinical use in a private hospital in Sao Paulo, Brazil

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Background: Human Cytomegalovirus (CMV) is a member of the Herpesviridae family. Primary CMV infection in healthy individuals is asymptomatic or results in a mild, non-specific illness. After acute infection, CMV establishes latent infection. Reactivation can occur in immunocompromised patients with an important morbidity and mortality in this group. Early diagnosis and CMV viral load monitoring in high risk patients are critical for an efficient infection management. To improve molecular diagnostic of CMV in our Clinical Laboratory, we validated the quantitative FOCUS CMV Simplexa kit in plasma samples.

Methods: Nucleic acids from plasma samples were extracted using automated EasyMag system and submitted to Real Time PCR with Focus Simplexa CMV kit, using the 3M Integrated Cycler equipment which is a nucleic acid amplification system based on a centrifugal micro fluidic platform. Validation was conducted according to CAP guidelines. We evaluated accuracy, linearity, precision (intra and inter-assay) and sensitivity. Accuracy was tested comparing obtained results with previous data. Linearity was determined using dilution series of a high viral load sample, while intra and inter-assay variations were determined using 4 pools of samples (high, medium, low-medium and low) and a negative plasma obtained from the blood bank. Sensitivity was established using samples with viral loads close to the detection limit of the kit.

Results: For accuracy, we compared the results of 53 samples and obtained a mean log difference of 0.21 Log copies/mL. Analytical linearity was evaluated in quadruplicates using the calibration CMV standard from FOCUS, ranging from 2,010,000,000 copies/mL (9.3 Log copies/mL) to 750 copies/mL (2.8 Log copies/mL). The correlation curve obtained had R^2=0.9993. Intra and inter-assay variation of all 4 pools of samples were lower than 10%, as expected. Finally, Detection limitwas tested with samples with 330, 153 and78 copies/mL. The test was able to detect viral load of 330 copies/mL with 100% confidence and 153 copies/mL with 90% confidence.

Conclusion: The results of validation demonstrated that the kit is reliable and useful for quantification of CMV in plasma samples, and combined with its fast turnaround time and decreased hands-on time, make this assay highly suitable for the rapid diagnostics of CMV infections in the clinical laboratory.

B-200

Sodium citrate at 8% is equivalent to EDTA as anticoagulant of choice for circulating cell-free DNA analysis: low contamination by blood cells genomic DNA and inhibition of blood nuclease activity.

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Background: Despite the intensive research, few circulating cell-free DNA (cfDNA) analysis have been translated to clinical practice. The lack of preanalytical consensus is a major obstacle. Traditionally, the EDTA is the anticoagulant of choice for studding cfDNA. Moreover, because of the lack of cell protection, the cfDNA is susceptible to blood nucleases, but the impact of these enzymes has long been neglected. Here, we studied the initial amount of cfDNA, it's stability and the blood nucleases activity in plasmas (EDTA, citrate, heparin) and serum samples.

Methods: Fresh blood from 20 health donors was collected simultaneously in K3EDTA, sodium citrate 3.2%, sodium heparin, and Z serum clot activator tubes (all from Greiner-bio-one). The citrate 8% samples were obtained by transferring fresh blood sequentially to 3 citrate 3.2% tubes. Serum or plasmas were generated within 10-15 minutes after the venipuncture. DNA extraction was performed by using Nuclisens easyMAG (Biomerieux). RNAse P was the target used for cfDNA quantification in a StepOne qPCR System (Life technologies) by using hydrolysis probe chemistry and absolute quantification. The results were shown as median in Genomic Equivalents/mL. Statistical analysis was Friedman's test. The cfDNA stability was evaluated treating (or not) the samples with 25U of DNAse I for 1h at 37°C before RNAse P assay. To investigate sample's nuclease activity a hydrolyze probe and a passive reference (ROX) were added to the crude samples and the fluorescence increase were measured for 24h at 37°C in the qPCR system. For nucleases inhibition assay a serial dilution of citrate (0.4 to 14%) was used.

Results: The cfDNA amounts in EDTA (158.7 GE/mL) and in citrate (130 GE/mL) were similar (p=0.27) and lower than the levels found in heparin (413 GE/mL; p=0.031-EDTA, p<0.0001-citrate) and in serum (815 GE/mL; p=0.0012-EDTA, p<0.0001-citrate). The nuclease activity was higher in heparin (arbitrary considered 100%), 90% in serum, 66% in citrate and not detected in EDTA. The nuclease activity curve in citrate was different from serum and heparin suggesting an inhibitory effect. The treatment with DNAse I reduced the cfDNA amount in EDTA by 1.1-fold, in serum by 1300-fold, in heparin by 242-fold and in citrate by 1.3-fold. In the citrate serial dilution experiment, no nucleases activity was detected from 7%. Increasing the citrate concentration to 8% did not change the initial cfDNA amount (96.86 GE/mL) compared to EDTA (129.2 GE/mL; p=0.12) and citrate (89.57 GE/mL; p=0.99). The nuclease activity was not detected in citrate 8% and treatment with DNAse I did not alter its cfDNA amount, reduction of 1.01-fold.

Conclusion: The citrate 3.2%, citrate 8% and EDTA have similar initial cfDNA, although lower when compared to heparin and serum. The nuclease activity was higher in heparin and serum, partially inhibited in citrate 3.2% and completely blocked in EDTA and citrate 8%. The divalent ions chelators citrate 8% and EDTA share a common mechanism of both avoid blood cells genomic DNA contamination to cfDNA and inhibit blood nucleases. The high-levels of cfDNA in serum and heparin should be attributed to the coagulation and direct lyses of blood-nucleated cells, respectively.

B-201

Development of a real-time PCR genotyping assay to detect HLA-B*5701 allele associated with abacavir hypersensitivity reaction

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Background: Abacavir sulfate is a nucleoside reverse-transcriptase inhibitor with potent antiviral activity against HIV. 5-10% of individuals being treated with abacavir develop a potentially life-threatening hypersensitivity reaction (ABC-HSR). Human leukocyte antigen (HLA) B*5701 allele strongly predicts ABC-HSR. Therefore, Pharmacogenetic screening for the HLA-B5701 allele is recommended prior to initiation of abacavir therapy. In San Francisco General Hospital, a two-color B57-specific immunofluorescence assay with FacsCanto flow cytometry was utilized to detect all HLA-B57 subgroups. Preliminary positive samples are sent to a reference

lab to identify true HLA-B*5701 positive patients. Recent studies have shown that a HCP5 single-nucleotide polymorphism (SNP), rs2395029, is in perfect linkage disequilibrium with the HLA-B*5701 allele: the sensitivity of the HCP5 SNP for carriage of the HLA-B*5701 allele was 100% and specificity was 99%. **Objective:** Develop an accurate in-house assay utilizing real-time polymerase chain reaction (PCR) and fluorescence monitoring. **Methods:** DNA extraction from blood samples was performed with a Qiagen DNA mini kit, and DNA concentration was measured using a NanoDrop ND2000. A rapid-cycle PCR was developed using the Rotor-Gene Q 2plex HRM system. Forward primer: GAGTGCCCATTGAACTACACA, reverse primer: GCTGGTCTCTGGACACATACTG, wild-type probe: FAM - AGCTGCCACAGGG - BHQ1 plus, mutant probe: CAL Fluor Orange 560 - AGCTGCCCCAGGG - BHQ1 plus. Thermocycling conditions were 20 sec at 95□C, followed by 40 cycles at 95□C for 3 sec and 60□C for 30 sec. PCR was performed in a 25-ul volume in the presence of 1X Taqman GTXpress master mix, 900 nmol/L of each primer, 250 nmol/L of each probe, 2 ul DNA, and DEPC H2O. 2-fold serial dilutions of a wild-type (T/T) sample and a mutant sample (G/G), with each dilution amplified in triplicates, were tested to evaluate the linearity and repeatability, as well as the limit of detection of the genotyping assay. A standard curve was constructed for each sample on the basis of DNA serial dilution, on which Ct values were plotted against the log value of the target DNA amount. Blood samples of 49 patients who were diagnosed of HIV were included in the patient comparison study between the new RT-PCR assay and PCR-SSOP method of the reference laboratory. **Results**: Ct values were obtained from amplification of serial dilutions of a wild-type sample from100 ug/ul to 3.125 ug/ul and a homozygous mutant samples from 10 ug/ul to 0.625 ug/ul, respectively. The regression equation of the wild-type sample was y=-3.4317x + 30.912, with a R^2 of 0.9985. The intra-assay coefficient of variation (CV) for all dilutions ranged from 0.037% to 0.73%. The regression equation of the homozygous mutant was y=-3.2123x+31.021 with a R^2 of 0.9907. CV for all dilutions ranged from 0.13% to 0.31%. Patient comparison study revealed that this real-time PCR assay demonstrated 100% sensitivity and 100% specificity when validated with 10 positive and 39 negative samples previously confirmed by the reference lab. **Conclusion:** A real-time genotyping assay was developed to identify positive and negative HLA-B*5701 alleles. This approach offers a sensitive, rapid and cost-effective screening assay prior to abacavir prescription. The genotyping assay has a wide dynamic range of reliable amplification linearity.

B-202

Development and Validation of a Clinical Sequencing Assay Using RNA-Seq to Direct Treatment of Relapsed Pediatric Cancers

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Background: Cancer continues to be the leading cause of death in children in the US. The aim of this study was to develop a clinically-validated, RNA-sequencing (RNA-Seq) assay of formalin-fixed, paraffin-embedded (FFPE) tumor tissue to detect actionable mutations and/or pathway activation in pediatric cancer relapse patients who have a <20% chance of event-free survival.

Methods: We studied FFPE tissue from 12 tumors previously identified to have gene amplification and/or over-expression. Purified RNA was quantified and evaluated by use of Nanodrop (2 - 3,000 ng/uL RNA), Invitrogen Quant-IT Qubit RNA Broad-Range Assay Kit (1 ng/uL - 1 ug/uL) and Agilent Bioanalyzer RNA Pico 6000 Kit (AMR 0.05 - 5ng/uL). RNA Integrity Numbers ranged from 2 - 4.3; 28S/18S values were 0 for the majority of samples. All purified samples were sonicated to ensure sufficient fragmentation of RNA. The first 4 samples were sequenced in multiplex on the Illumina MiSeq platform, and manual analysis of overexpression was performed. Within-sample normalization of genes of interest was accomplished by selecting 9 housekeeping genes expressed in all samples. When matched normal tissue was unavailable, data from The Cancer Genome Atlas (TCGA) database was used for comparison with our results.

Results: At abstract submission, 4/12 specimens have been fully analyzed: dedifferentiated liposarcoma of kidney, invasive ductal carcinoma of breast, B-cell lymphoma metastasized to tonsil, and lung adenocarcinoma metastasized to lymph node. Specimens contained known gene amplification of MDM2, HER2 (ERBB2), or MYC, or an EGFR mutation, respectively. RNA transcript over-expressions of MDM2 (177-fold), HER2 (20-fold) and EGFR (7-fold) were detected by manual analysis in tumors with matching gene amplification or mutation. Number of reads ranged from 3.5 - 7.3 million per sample, and thus coverage was insufficient to produce reads in exons 19-21 of the EGFR gene in order to detect intragenic mutations or deletions. Therefore only one specimen per flow-cell was sequenced in subsequent runs, achieving approximately 25 million reads per sample. At time of abstract submission, the TCGA database had no normal control data of the tissue type for the MYC sample analysis; MYC data will be analyzed with a bioinformatics pipeline in development that will define approximately 100 suitable housekeeping genes for normalization.

Conclusions: We describe a proof-of-principle for a clinically validated whole-transcriptome RNA-Seq assay from archival FFPE tumor tissue in order to detect overexpression of clinically relevant genes in cancer patients.

B-203

Diagnositc yield of chromosomal microarray for individuals with developmental disabilities or congenital anomalies.

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Chromosomal microarray (CMA) is increasingly utilized for genetic testing of individuals with unexplained developmental delay/intellectual disability (DD/ID), autism spectrum disorders (ASD), or multiple congenital anomalies (MCA). Guidelines recommend the use of CMA as first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies.

OBJECTIVES To implement an algorithm for array comparative genomic hybridization (aCGH) testing in patients with unexplained unexplained DD/ID, ASD, or MCA. To assess the diagnostic yield of aCGH for individuals with developmental disabilities or congenital anomalies and compare it to the literature data.

METHODOLOGY We performed, since 06/2009, aCGH to 161 patients referred from the Neuropediatrics department of our hospital with unexplained DD/ID, ASD, or MCA. DNA was extracted with an automated method, QIAamp DNA Blood Mini Kit in a QIACube instrument (QIAgen). aCGH was performed in the beginning with Nimblegen CGH ISCA Plus 6x630K arrays (Roche Diagnostics) and since its exit from the microarray business with the Signature Genomics CGX-HD 4x180K arrays (Perkin Elmer). Both probes design follow the ISCA consortium guidelines (International Standards for Cytogenomic Arrays). Results were reported following ISCN 2013 recommendations. If a CMA variant was observed, parental samples were analyzed to assess whether it is a de novo or an inherited alteration. Copy number variations are assigned the following interpretations: Abnormal (well established syndromes, de novo variants and large changes); VOUS (variants of unknown significance) and likely benign (not previously reported but inherited from a healthy parent). Diagnostic yield was defined as the number of patients with abnormal variants divided by the total number of patients tested.

RESULTS 161 patients and 42 parents were studied. 110 of the 161 patients (68,32%) had a normal aCGH result. 51 patients (31,68%) showed an abnormal result. After analyzing every single case and performed parental tests, we classified the alterations as follows: 21 abnormal (13,04%), 7 likely benign (4,35%), 1 VOUS (0,62%) and 9 are still pending parental aCGH results (5,59%). Abnormal variants deletion or duplication size varied from 200 Kb to 8 Mb. The diagnostic yield, calculated as 21 abnormal patients divided 161 patients, is 13,04%.

CONCLUSIONS aCGH showed a much higher diagnostic yield than conventional cytogenetics techniques for the diagnosis of unexplained unexplained DD/ID, ASD, or MCA. These figures are according to the literature. Our results could be even higher as there are still 9 cases pending parental aCGH results, so these variants can be reclassified as abnormal or likely benign. The use of CMA as a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies has proven to surpass the classical approach with conventional cytogenetics. The use of CMA is cost effective in a child with DD/ID, ASD, or MCA. CMA is not inexpensive, but the cost is less than the cost of a G-banded karyotype plus subtelomeric FISH plus other techniques such as MLPA, and the yield is greater.

B-205

A retrospective analysis of Western Blot findings and subsequent Nucleic Acid Amplification Testing of samples with Immunoassay Screening positive for Human Immunodeficiency Virus

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Background: With the first clinical observation of Acquired Immune Deficiency Syndrome (AIDS) in 1981, the entire medical landscape pertaining to the diagnosis, treatment and monitoring of an infection by the Human Immunodeficiency Virus (HIV) has progressed continuously. The western blot assay is the current method of choice for confirming HIV results positive by immunoassays. We compared Western Blot outcomes against viral load quantification on immunoassay-positive samples. A total of 70 anonymised samples that were screening-positive on the Roche HIV

Combi or Combi PT immunoassay (Roche Diagnostics, Switzerland) were sent for western blot confirmation and also tested for viral load using the improved Roche Cobas Taqman HIV1 Monitor version 2 (Roche Diagnostics, Switzerland).

Samples are determined to be western blot positive if any 2 bands for p24, gp41, gp120/160 or 2 of 3 envelope bands with or without Group Antigen and/or polymerase bands are present, as defined by Centres for Disease Control and Prevention and World Health Organisation criteria respectively. Indeterminate results are defined as the presence of bands that do not meet positive criteria while inconclusive findings are those that do not fit negative, positive or indeterminate criteria.

Methods: A total of 84 anonymised samples were screened for infection by the Human Immunodeficiency Virus, using the Roche Diagnostics immunoassay HIV Combi or Combi PT. Seventy samples were determined to be at the grey zone cut-off index of 0.9 or greater, indicating presumptive positivity. These samples were subsequently sent for western blot analysis at the National Reference HIV Laboratory with split samples tested using the HIV Monitor v2 on the Roche Taqman 48.

Results: Of 84 samples tested, 14 samples were determined to be screening-negative with no viral RNA detected by nucleic acid testing. Thirty-four samples were screening-positive and subsequently confirmed by western with viral loads of log 1.53 to 6.00 copies per milliliter. Two samples were defined as western blot indeterminate, with no virus detected, while the remaining 7 indeterminate samples and 1 inconclusive sample had viral loads of log 5.33 to 6.48 copies per milliliter. The mean and standard deviation of viral loads for western blot non-positive and non-negative samples were 6.17 and 0.44 respectively. Western blot positive samples yielded a lower average value of log 4.62 copies per milliliter with a standard deviation of 0.73.

Conclusion: While there is a robust concordance between definitive western blot outcomes and nucleic acid testing, the majority of results classified as 'inconclusive' and 'indeterminate' were associated with significant viral loads. This is not unexpected as the western blot is an antibody assay. Re-testing is usually recommended at least 4 weeks after the first result for these patients. Thus, from a clinical perspective, treatment with anti-retroviral prophylaxis in these patients may potentially be delayed until such time when the western blot turns positive with full seroconversion, possibly leading to a sub-optimal outcome for these patients.

B-206

Development and evaluation of a new molecular diagnostic method for HER2 testing

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Background: HER2 PCR Testing results have been published for decades with favorable concordance with IHC and FISH testing results. However, on the ground of "insufficient evidence", current guidelines still exclude PCR-based method from determining HER2 status. To find a remedy, we surveyed and found that the incompatible and insufficient evidence was primarily due to the lack of consistency in PCR assay design. Here, we propose a standardized HER2 RT-PCR assay with proven reproducibility for adoption with the hope that a standardize HER2 RT-PCR testing will gain the traction in creating more compatible data toward achieving the goal of having ASCO/CAP to include HER2 RT-PCR assay in HER2 testing guideline.

Methods: One-step RT-PCR with external calibrators was utilized to quantify HER2 RNA copies in samples. To evaluate the performance and concordance between RT-PCR and FISH test, breast invasive ductal carcinoma (IDC) or adjacent normal specimen was collected and processed into FFPE or OCT sample immediately after biopsy or surgery. RNA samples were determined to obtain optimal final concentration of 25μg/mL. HER2 positive cutoff value was established by conducting a statistical population study on tumor versus adjacent normal samples.

Results: The intra- and inter-run coefficient of variations of the assay were <5% (in terms of concentration in $\log_{10}$). The dynamic range of the assay was between $5.4x10^3$-$5.4x10^{12}$ copies/mL (R^2=0.99). Moreover, around 85% overall percentage agreement (OPA) was obtained when compared with FISH test with FFPE or OCT samples.

Conclusion: A standardized RT-PCR assay and user procedure presented here can produce consistent test results regardless of the types of patient samples and has high potential to be included in the ASCO/CAP HER2 testing guideline.

HER2 RT-PCR versus FISH test in NCKU hospital			FISH test			Agreement Score		
			Positive	Negative	Sum	PPA	PNA	OPA
HER2 RT-PCR test	FFPE samples	Positive	16	3	19	80%	89%	85%
		Negative	4	24	28			
		Sum	20	27	47			
	OCT samples	Positive	8	2	10			
		Negative	2	16	18			
		Sum	10	18	28			
PPA: Positive Percentage Agreement; PNA: Negative Percentage Agreement; OPA: Overall Percentage Agreement								

B-207

Real-time NASBA Targeting HPV E6/E7 mRNA Overcomes Low Specificity of HPV DNA Test

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Background: Cervical cancer is one of the most common cancers in women, and ranks second by female cancer mortality rate. About 40 of human papillomaviruses (HPV) can infect the cervix and are divided into high- and low-risk groups based on their frequent detection in carcinoma or low-grade lesions, respectively. Persistent infection with these types is the major risk factor for the development of cervical cancer. So it was recommended HPV genotyping test as complementary diagnosis. But, HPV DNA was detected both in cancerous tissues and in normal tissues according to cytological diagnosis. Several studies have suggested that detection of E6/E7 oncogene transcripts of high-risk HPV types would provide higher specificity in screening for the risk of development of high-grade of cervical samples. The synergistic effects of E6 and E7 proteins results in disturbance of cell cycle regulation, prevention of apoptosis, and, ultimately, transformation and survival of neoplastic and dysplastic cells.

Methods: This study aims to evaluate the clinical performance of the commercial kit targeting HPV E6/E7 mRNA and compare it with HPV DNA genotyping for the detection of high-grade squamous intraepithelial lesions (HSIL) and cancer in a Korean population. NucliSENS EasyQ HPV kit (bioMérieux, Marcy, France) using a nucleic acid sequence-based amplification (NASBA) technique is chosen. This assay utilizes molecular beacon probes for real-time detection and typing of E6/E7 mRNA from HPV genotype 16, 18, 31, 33 and 45.

Results: HPV DNA tests were positive in 100% and 53% of abnormal (SCC, HSIL, ASC-H, LSIL, and ASC-US) and normal cytology cases, respectively. Positivity rates of HPV E6/E7 mRNA assay were 75%, 74%, 60 %, 56%, 29% and 9% for SCC, HSIL, ASC-H, LSIL, ASC-US, and normal cases, respectively. The data from this study seems clearly show that the real-time NASBA for diagnosis of cervical cancer has higher clinical specificity than HPV DNA genotyping test, since the positivity rate detected by the real-time NASBA in normal samples was significantly lower than that detected by the HPV DNA genotyping test. However, the positive rate detected by the real-time NASBA in cancerous samples was much lower than that detected by the HPV DNA genotyping test. In this respect, the sensitivity of the real-time NASBA for cervical cancer diagnosis seems to be lower than current HPV genotyping test. The main reason of this low sensitivity was primarily due to the different prevalence of the high-risk cervical cancer causing HPV genotypes of Korea.

Conclusion: Data from the current study suggests that the NucliSENS EasyQ HPV E6/E7 assay (bioMérieux) has higher specificity than DNA assays, and can overcome DNA assays' shortcoming of low specificity in clinical detection of high-grade cervical lesions and ability to predict the risk of their development in HPV-infected women. Expanding the types of HPV targeted by mRNA assays may increase sensitivity as well as specificity of detection of high-grade cervical lesions.

B-208

Bioinformatics analysis to determine prognostic mutations of 72 de novo acute myeloid leukemia cases from The Cancer Genome Atlas (TCGA) with 23 most common mutations and no abnormal cytogenetics

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Objective: Acute myeloid leukemia (AML) is a heterogeneous malignancy with many described karyotypic and molecular abnormalities. Recurrent karyotypic abnormalities in AML have been well established for treatment. However, approximately half of AML patients have no karyotype abnormality (CN-AML). An important issue in the treatment of AML is how gene mutation patterns may help physicians guide the management of patients in daily practice. The Cancer Genome Atlas (TCGA) database has available data from 200 cases of de novo AML including cytogenetics, 260 gene mutations, and survival duration for each case. As previously reported, in this database a total of 23 genes were significantly mutated and another 237 were mutated in two or more samples. We utilize clustering analysis on this database to correlate the presence of 23 common mutations with the prognosis of de novo CN-AML cases.

Methods: Cases with positivity for the most common 23 mutations and no cytogenetic abnormalities were selected from the TCGA. Unsupervised neural network analysis with NeuroXL Clusterizer (OLSOFT LLC, Moscow) was performed on these cases to group them into clusters according to their pattern of mutations and survival duration. The next step was to find interaction between wild-type genes, gene mutations (with their associated proteins) and common chemotherapy to gain more insight into response of CN-AML patients to treatment, which was achieved with Ingenuity IPA software (Ingenuity Systems, Inc., Redwood City, CA).

Results: 121 cases with positivity for the 23 most common mutations were obtained from the original set of 200 AML cases. Subsequently, 72 cases with no cytogenetics abnormalities (CN-AML) were obtained from these 121 cases. Within the 72 CN-AML cases, the following mutations were not present: TP53, NRAS, KIT, EZH2, and HNRNPK, leaving 18 mutations in this subset of patients. Using appropriate threshold for mutation frequency (75%), clustering was found to be based on only 4 mutations: NPM1, FLT3-ITD, RUNX, and DNMT3A. The following prognostic groups were found: (a) good: NPM1, CEBPA, or TET2, (b) intermediate: NPM1/DNMT3A, or other mutations, (c) poor: RUNX1, FLT3-ITD/NPM1, FLT3-ITD/CEBPA, or FLT3-ITD. Pathway analysis revealed significant causality between the mutations FLT3-ITD, NPM1, DNMT3A, IDH2, RUNX1, TP53, KIT, and CEBPA and the chemotherapy agents cytarabine, idarubicin, fludarabine, topotecan, etoposide, hydroxyurea, dexamethasone, methotrexate, and decitabine.

Conclusions: Combinations of mutations appear to dictate the clinical behavior of AML in terms of prognosis. This study provides further molecular characterization and prognostic data for the heterogeneous group of CN-AML patients.

B-209

Validation of a genetic test for lactose tolerance in a Brazilian hospital

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Background: Lactose, the predominant carbohydrate in breast milk, is hydrolyzed in the intestinal mucosa by lactase-phlorizin hydrolase (LPH). Lactase is produced during the weaning and undergoes a physiological decline in adult, which leads to lactose intolerance symptoms. In some populations LPH activity persists throughout life, a condition known as adult lactase persistence. The conventional diagnostic test is performed challenging the patient with lactose, which can cause diarrhea and abdominal pain in intolerant patients. Several studies have shown that two single nucleotide polymorphisms in the introns of a helicase (MCM6) upstream the lactase gene [rs4988235 (LCT-13910C/T) and rs182549 (LCT-22018G>A)] correlate with lactose intolerance through differential transcriptional activation of the lactase promoter. Intolerance genotype is a recessive trait in both SNPs: CC-13910 and GG-22018.

Methods: In order to offer a molecular test that will not need lactose consumption, we developed two Real Time PCRs reactions to target those SNPs. DNA was isolated from peripheral blood using EasyMag Extractor (Biomeriéux) and submitted to Real Time PCR using TaqMan® SNP Genotyping Assay kits (Applied Biosystems) with probes specific for each SNP.

Results: During validation, we tested 59 samples with previous conventional test results and found a 91.5% correlation and 100% reproducibility of all possible genotypes. Considering that lactose tolerance prevalence in different populations varies greatly, we determined the genotype frequency of 511 patients in our service and found a frequency of 51.86% CC, 41.49% CT and 6.65% TT for LCT-13910 and 51.66% GG, 40.70% GA and 7.63% AA for LCT-22018.

Conclusion: Molecular test was implemented in our Clinical Laboratory in November, 2012 and since then has been an excellent option for patients instead of the conventional test. Approximately, half of tested patients were intolerant to lactose. Those data are in accordance with previous Brazilian studies, and may reflect the great admixture in Brazilian population.

B-210

Genotyping of drug-metabolizing enzymes CYP2D6, CYP2C19, CYP2C9, CYP3A4 and CYP3A5 in patients prescribed pain medications

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Background: The cytochrome P450 (CYP450) superfamily represents a group of enzymes which are associated with the metabolism of more than 90% of human drugs. To date, a large number of allelic variants as well as copy number variations (CNV) have been reported and the number of alleles is still growing. Many genetic polymorphisms within the CYP450 superfamily result in altered enzyme expression or activities, significantly effecting drug metabolism. Based on the genotypes of the CYP450 enzymes, patients may be categorized as ultra-rapid metabolizer (UM), extensive metabolizer/normal metabolizer (EM), intermediate metabolizer (IM), and poor metabolizer (PM). Even though the value of routine pharmacogenetic testing is still debated, accumulated evidence indicates that genotyping drug-metabolism enzymes will help physicians to tailor pain treatment to individual patients.

Methods and Materials: DNAs were extracted from buccal swabs collected from 200 patients prescribed pain medications. Five genes in CYP450 superfamily: CYP2D6, CYP2C19, CYP2C9, CYP3A4, and CYP3A5, were genotyped. Genotypes of the 5 genes were reported using the star (*) allele nomenclature. 16 alleles (*2, *3, *4, *5, *6, *7, *8, *9, *10, *11, *12, *14, *15, *17, *29, *41) and copy number of CYP2D6; 10 alleles (*2, *3, *4, *5, *6, *7, *8, *9, *10, *17) of CYP2C19, 2 alleles (*2, *3) of CYP2C9, 6 alleles (*1B, *2, *3, *12, *17, *22) of CYP3A4, and 8 alleles (*1D, **2, *3, *3B, *6, *7, *8, *9) of CYP3A5 were tested. Allele *1 is assigned by default when no other listed alleles are detected. Phenotypes were assigned based on the guidelines from the clinical pharmacogenetics implementation consortium (CPIC) and/or published general rules: 1) UM: more than 2 copies of functional alleles; 2) EM: 2 copies of functional alleles, or 1 copy of functional allele and 1 copy of decreased functional allele; 3) IM: 2 copies of decreased functional alleles, or 1 copy of functional and 1 copy of non-functional alleles, or 1 copy of non-functional and 1 copy of decreased functional alleles; 4) PM: 2 copies of non-functional alleles.

Results: Among the 200 specimens, 11 allelic variants (*2, *3, *4, *5, *6, *9, *10, *11, *17, *41) in CYP2D6, 3 allelic variants (*2, *9, *17) in CYP2C19, 2 allelic variants (*2, *3) in CYP2C9, 3 allelic variants (*1B, *3, *22) in CYP3A4, and 4 allelic variants (*1D, *2, *3, *6) in CYP3A5 were detected. The most common genotypes and phenotypes are: 1) for CYP2D6: *1/*2 (EM, 20%), *1/*4 (EM, 13.5%), and *1/*41 (EM, 10.5%); 2) for CYP2C19: *1/*1 (EM, 38.5%), *1/*17 (UM, 27%), and *1/*2 (IM, 19%); 3) for CYP2C9: *1/*1 (EM, 67.5%), *1/*2 (EM, 22.5%) , and *1/*3 (IM, 8%); 4) for CYP3A4: *1/*1 (EM, 80.5%), *1/*1B (EM, 10%); and *1/*22 (EM, 4.5%); and 5) for CYP3A5: *3/*3 (PM, 76.5%), *1/*3 (IM, 16%), and *3/*6 (PM, 1.5%).

Conclusions: The frequencies of allelic variants and genotypes we found in the five genes are in accordance with preciously published results. In our targeted population, the most common allelic variant found was the non-functional *3 allele in CYP3A5, with a frequency of 86%.

B-211

Development of an LNA-Blocker enhanced, allele-specific, loop-mediated isothermal amplification (AS-LAMP) method for detection of single base mutations in beta-thalassemia patients

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Background: It was found that five mutations, namely 654M, 41/42M, 28M, 17M and 27/28M, are the most commonly occurring single base mutations in beta-thalassemia patients in China. Being able to detect and distinguish these mutations is important for patient screening and for diagnosis of the disease. The loop-mediated isothermal amplification (LAMP) method is known to be a rapid (complete in less than 45 min) and simple way for detection of gene deletion and insertion events, but is not specific

enough to distinguish single base difference. This study was aimed to improve the sensitivity of LAMP method on single base mutation detection in beta-thalassemia patients by using Locked nucleic Acid (LNA) modified primers in the reaction.

Methods: The LNA-Blocker enhanced, allele-specific, loop-mediated isothermal amplification (AS-LAMP) was carried out under isothermal condition. To enhance the specificity of AS-LAMP, an LNA modified allele-specific primer and an LNA modified wild type blocking primer were used in the reaction. LNA is a high affinity DNA analogue that has increased target specificity and high melting temperature. This method was used to detect and distinguish single base mutations in beta-thalassemia patients. This method was validated with positive and negative controls and with 145 clinical samples of patient genomic DNA (46 positive samples containing all 5 mutations and 99 negative samples).

Results: The LNA-Blocker enhanced AS-LAMP method showed high specificity with either plasmid or genomic DNA targets in reactions. The assay had a detection limit of approximately 100 copies of the target sequence (95.7% in sensitivity, 44/46), comparable to the traditional AS-LAMP. However, the specificity of LNA-Blocker enhanced AS-LAMP (96.0%, 95/99) was much higher than the traditional AS-LAMP (75.8%, 75/99). Multiplex detection of all 5 targets was also achieved with LNA-Blocker enhanced AS-LAMP.

Conclusion: LNA-blocker enhanced AS-LAMP provides a highly specific isothermal method for detection of single mutations and for screening of single mutations in beta-thalassemia patients.

B-212

Detection of fetal aneuploidies by quantitative fluorescent polymerase chain reaction in the Brazilian population

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Background: Aneuploidy occurs from non-disjuntion during gametogenesis and results in an abnormal number of chromosomal in the gametes. The gamete joined in fertilization contains an extra copy of one chromosome or one of the chromosomes is missing. These alterations can results in trisomy or monosomy in the fetus or embryo which are frequently associated with severe congenital abnormalities, mental retardation and shortened life expectancy. The majority of first trimester abortion cases is caused for aneuploidies. Quantitative fluorescent polymerase chain reaction (QF-PCR) is a method which has been regularly used for the diagnosis of common chromosomal abnormalities in recent years with low error rates. Objective: To determine the frequency of the most common chromosomal aneuploidies causing abortion in Brazilian population. Methods: The study is the retrospective statistical analyses of data registered on 70 women submitted to molecular studies of chromosome disorders at the Hermes Pardini Institute, Belo Horizonte, Minas Gerais state, Brazil in 2013. All patients had spontaneous abortion and the ovular remains, embryo tissue were analyzed after the DNA extraction. Was compared the genetic profile of the sample with the maternal blood, a differential that allows to detect maternal contamination in the sample, improving the reliability of the results. Contaminated samples were excluded from the study. After that all samples were tested using quantitative fluorescent polymerase chain reaction (QF-PCR) for detection or exclusion of aneuploidy in chromosomes 13, 15, 16, 18, 21, 22, X and Y (Chomo Quant ®QF-PCR Kits). Results: The age average of the patients was 34.4 ± 4.7 years old. We detected 44 subjects (62.9%) with aneuploidies or euploidies. Nine cases of trisomy 15 (12.9%), nine cases of trisomy 22 (12.9%), seven cases of monosomy X (10.0%), four cases of trisomy 13 (5.7%), four cases of trisomy 16 (5.7%), four cases of trisomy 21 (5.7%), five cases of triploidy (7.1%), a case of trisomy 18 (1.4%) and a case of paternal isodisomy (1.4%) were detected by QF-PCR. Conclusions: Quantittaive Fluorescent PCR is a robust and accurate method for rapid aneuploidy detection. One the first suspicions of spontaneous abortions is numerical abnormalities in 13, 18, 21 and sex chromosomes, representing 22.8% of our cases of aneuploidy. But the results show the importance of also being analyzed changes in chromosomes15, 16 and 22 which concentrated 31.5% of the aneuploidies detected. We are going to analyze a larger number of samples in order to confirm the importance of study chromossomes 15, 16 and 22 disorders.

B-214

Detection of Von Hippel - Lindau (VHL) Gene Copy Number Variations Using Digital Droplet PCR

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Background: Our current assay for detection of large deletions in the VHL gene uses a combination of MLPA based probes (MRC-Holland b.v) and Luminex® FlexMap technology. It is a two-day assay that requires 400ng of DNA input, and consists of overnight probe hybridization, amplification, bead hybridization and Luminex detection. Due to these complex workflows, assay failures are not rare. Digital droplet PCR (ddPCR) might represent a faster, more sensitive and more reliable alternative. DdPCR is based on traditional PCR amplification and fluorescent probe-based detection methods, but partitions each reaction into multiple nanodroplets. Quantitation is based on counting the proportion of droplets that show amplification. Poisson statistics are then applied to back calculate the copy number in the original sample. This allows for highly sensitive and reproducible absolute quantification of nucleic acids without the need for standard curves.

The objective of this study is to develop a method for detection of single or multiple exon deletions in VHL using digital droplet PCR (ddPCR).

Methods: Prior to PCR cycling, reactions are prepared as for traditional fluorescent qPCR methods. Fluorescent probes and two primer sets encompassing 80-120bp of each VHL exon are added to each reaction mixture, which also includes an additional reference gene. The final reaction mix is then partitioned into thousands of nano-droplets. Ideally, the starting DNA concentration is such that this will result in a mixture of droplets containing either zero or one template molecule per droplet. The droplets are pooled back into a PCR tube, and cycling is performed. At the conclusion of cycling, negative and positive droplets are counted in a flow cytometer like device. Total assay time is approximately three hours.

Various input concentrations of primers, probes and DNA were tested during validation. Intra- and inter-assay concordance of copy number assessment was tested on three different DNA specimens with and without deletions. Patient specimens containing deletions of one, two, or all three exons of the VHL gene were used for method comparison between MLPA and ddPCR. Finally, formalin fixed, paraffin embedded (FFPE) specimens, which habitually fail MPLA, were re-tested by ddPCR

Results: Primer and probe concentrations and DNA input were optimized and standardized for the method, with 10ng of DNA input required for successful copy number estimation. A direct comparison of TaqMan probes with 3'-MGB-labeled probes showed that MGB-labeling provided better specificity. Copy number assessment concordance was 100% between runs. The method comparison showed 100% concordance between MLPA and ddPCR. The FFPE specimens were successfully assayed by ddPCR while failed testing by MLPA.

Conclusion: DdPCR for copy number estimation of the VHL gene variants is fast, reliable and accurate. It requires minimal nucleic acid input, with a 40-fold reduction of input DNA compared to MLPA. Because of this advantage, difficult specimen types, including FFPE tissue, are now capable of being characterized. Additionally, same-day results are available with the ddPCR method, reducing total run-time from 48- to 3- hours.

B-215

Absolute Quantification of Graft derived cell-free DNA (GcfDNA) early after Liver Transplantation (LTx) using Droplet Digital PCR

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Background: The diagnostic value of GcfDNA as measure of graft integrity after LTx has been recently proven [1,2]. The yin and yang of using percentage values vs. absolute GcfDNA quantification is, nevertheless, under discussion [3]. Where the ratio of graft to host cfDNA has analytical advantages by eliminating disturbing variables, such as DNA extraction efficiency, variabilities in host cfDNA may obfuscate the view on the engrafted organ. The early phase after LTx was used as model to interrogate whether the percentage or absolute plasma concentration of GcfDNA is a more valuable graft integrity measure.

Methods: GcfDNA percentage was determined by droplet digital PCR (ddPCR - Bio-Rad) as described [1]. A synthetic sequence of non-human origin (average length

of cfDNA) was spiked into 1mL plasma, and quantified with ddPCR after DNA extraction in one fluorescent channel. The total cfDNA was quantified using two combined human genomic dPCRs in the second channel as copies/mL (cp/mL). Total cfDNA was calculated using the spike-in without being extracted to assess the DNA extraction efficiency in each batch. GcfDNA concentration was defined as of the total cfDNA(cp/mL) x GcfDNA%. Plasma obtained during the first 10 days after LTx from 15 patients (one split-LTx) was investigated

Results: Ten repeated extractions of the same plasma pool from healthy volunteers yielded an average of 1069 diploid genomic cp/mL plasma with a CV of 7.5%. Of 185 samples six showed a low (<50%) extraction efficiency; the remainders had an average of 67%+9%. The total cfDNA was highly variable peaking at 6hr after reperfusion ($3.9x10^5$±$2.0x10^5$cp/mL) weaning to $1.3x10^5$±$0.9x10^5$cp/mL at day 10. The respective GcfDNA was $3.1x10^5$±$1.8x10^5$cp/mL (6hr) and $1.5x10^3$±$0.9x10^3$cp/mL (day10). The correlation between GcfDNA% and GcfDNA(cp/mL) values was weak (r=0.61;p<0.05). A comparison of the AUC (day1-day5) of AST with GcfDNA percentage and concentration showed a better association with absolute GcfDNA (r=0.65;p<0.05) compared to percentages (r=0.31;p=0.27). The initial half life was 1.3±0.6days for GcfDNA(cp/mL) and 2.9±1.6days GcfDNA(%), compared to 2.6±1.2days for AST.

Conclusion: A robust and precise ddPCR method for absolute quantification of GcfDNA, was developed, combining the analytical advantages of graft/host ratio (e.g. eliminating possible bias from interferences), with a robust quantification of total cfDNA. The GcfDNA concentration seems better associated with AST-values early after LTx and showed more rapid dynamics than GcfDNA percentage. Even though the initial post Tx phase, with highly variable amounts of total cfDNA, is particularly complicated, this method may also provide a better view on graft integrity in other situations, where the host cfDNA is increased due to non-transplantation related causes. As to whether the clinical utility is improved compared to percentage values for stable patients as well, is subject to further investigations.

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B-220

Frequency of G1691A (FV), G20210A (FII), C677T, A1298C (MTHFR), C282Y, H63D E S65C (HFE) among Brazilian blood donors and evaluation of OpenaArray method for SNP detection.

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Background: OpenArray® is a new PCR technology that uses a microscope plates and allow the detection of many SNPs at the same time.

Objective: We have used this technology to describe the genotypic frequencies of some SNPs related to venous thrombosis (G1691A and G20210A), to hyperhemocistinemia conditions (C677T, A1298C), and to hereditary hemochromatosis (C282Y, H63D and S65C), among Brazilian blood donors.

Methods: We tested 400 blood samples from Fundação Pró-Sangue Hemocentro of São Paulo, Brazil. The DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA concentration and quality was determined by spectrophotometry method using NanoDrop 2000 (Thermo Scientific, Wilmington, DE). The OpenArray platform (Life Technologies, Foster City, CA) was used to genotype the samples through TaqMan Genotyping detection system. The reproducibility of this method was evaluated running nine samples in triplicate in three different assays. The DNA samples were also tested using TibMol (TibMol, Berlim, Germany) reagents at Light Cycler 2.0 (Roche) plataform, with FRET as detection system, to compare the results and determine the accuracy of Open Array method. The genotypic frequency for each SNP was performed using by Taqman Genotyper v1.3 software (Life Technologies)

Results: Of 400 samples, 392 showed valid results. The results showed agreement of 100% for all assays tested, exception to HFE C282 (95.5% agreement). The only discordant sample result for HFE C282Y was confirmed by direct sequencing which showed that OpenArray result were correct. The calculated frequencies of each SNP found were FV G1691A 98,8% (G/G), 1,2% (G/A); FII G2021A 99,5% (G/G), 0,5% (G/A); MTHF C677T 45,5% (C/C), 44,8% (C/T), 9,8% (T/T); MTHF A1298C-60,3% (A/A), 33,6% (A/C), 6,1% (C/C); HFE C282Y 96,0%(G/G), 4,0%(G/A), HFE H63D 78,1%(C/C), 20,3%(C/G), 1,6% (G/G); HFE S65C 98,1% (A/A), 1,9% (A/T). The reproducibility test showed 100% of concordance among the replicates.

Conclusion: The Open Array genotyping method was used to detect simultaneously 7 different mutations in thrombophilia, folate and hemochromatosis related genes. Compared to other genotyping methods such as PCR-RFLP and sequencing, this method is, easy to perform and useful for high-throughput routines. The results found describe the frequency of SNPs related to diseases not well established by previous literature for Brazilian population. They are important to highlight the genetic profile of Brazilian blood donors.