Tuesday, July 28, 2015

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

Immunology

A-205

Application of Assay Appropriate Statistical Analysis Dramatically Improves Turn Around Time

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Background: Antibody responses to *Streptococcus pneumoniae* are often measured as an assessment of response to vaccination, particularly for patients with suspected immunodeficiency. The concentrations of antibodies are a function of each patient's immunological response to the vaccine; results between patients and between serotypes for the same patient can vary widely. To account for this, our method includes a series of dilutions for each patient, with the final result based on the weighted average of the dilutions. Unfortunately, this is a time-consuming process, taking up to 3 hours for a 54 patient load. The purpose of this study was to find an alternate method for calculation of the weighted average that would allow for improvement in turn-around-time, while maintaining analytical accuracy. Methodology: *Streptococcus pneumoniae* antibodies are measured by dispensing 5 serial dilutions of each patient sample into a 96-well vacuum filter plate. A mixture of Streptococcal polysaccharide-conjugated microspheres (representing each of the 23 polysaccharides in the Pneumovax-23 vaccine) is added to each patient dilution. Following incubation and washing, a fluorescein-conjugated anti-hIgG is added to each dilution. After a second incubation and wash, the mean fluorescence intensity (MFI) for each bead is measured on a Luminex platform. Calculation of patient results is performed using the statistical analysis program StatLia (Brendan Scientific). The MFI for each bead is compared to the MFI of the standards via a 5PL weighted regression, which yields a concentration for each of the 5 dilutions. The final result for each serotype, taking into account the results from each dilution, is determined through a series of weighting, curve fitting, and parallelism calculations. An alternate to StatLia was identified at http://www.itl.nist.gov/div898/handbook; this approach relates the weight as a function of the variability of the response at each standard (W=1/StdDev) A weighting function is obtained that can be related to the MFI at each of the standards. These are plotted Weight(Y) vs MFI(X) and fitted with a power equation (y=cx^b) to obtain a function that will calculate the appropriate weight to assign each of the 5 results. *Streptococcus pneumoniae* antibody results from patients (n=1122) were calculated using both methods and the results for each serotype were compared by linear regression. Results: Linear regression analysis comparing results calculated by StatLia and the new weighting algorithm showed slopes ranging from 0.836 to 1.060 for the 23 serotypes, with correlation coefficients ranging from 0.865 to 0.998. Overall qualitative concordance ranged from 96.8 to 99.2%. The amount of time required to calculate a 54 patient load dropped from 3 hours with Statlia to 2.8 minutes for the new weighting algorithm (N=4).The average turnaround time has improved from 4.8 days to 3.4 days for a representative 25 loads timed before and after the conversion. Conclusion: By researching alternate method of statistical analysis our laboratory was able to improve the turnaround time by more than 1 day while maintaining high quality results. Laboratories should look at their processes and evaluate them on a regular basis to determine if efficiencies can be gained.

A-206

Analytical Validation of an ELISA for the Quantitation of Calprotecin as a Marker of Intestinal Inflammation.

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Background: Fecal calprotectin has emerged as a promising marker for organic diseases of the bowel, including inflammatory bowel disease (IBD). This protein is produced by neutrophils, which are recruited to the intestine during a localized immune response. Upon activation, calprotectin is released, eventually entering the intestinal lumen. The amount of calprotectin present in feces is proportional to the amount of intestinal inflammation. The purpose of our study was to validate an ELISA for the quantification of calprotectin in feces for evaluation of suspected IBD. **Methods:** Fecal samples from patients (n=38, 18-70 years) with suspected or confirmed IBD and healthy donors (n=20, 27-82 years) were collected. Calprotectin was extracted by diluting between 80-120 μg of feces 1:50 with extraction buffer. The mixture was then homogenized and centrifuged. Finally, the supernatant was poured off, aliquoted, and frozen at -20°C until testing. Calprotectin was quantitated using the QUANTA Lite® Calprotectin ELISA (INOVA Diagnostics, Inc.). All experiments were performed manually; end-point colorimetric determinations were read on the Synergy H1 Hybrid plate reader (Bio-Tek). Data analysis was performed using Gen 5 software (Bio-Tek). The packet inserts were followed with no deviations. For validation, intra- and inter-assay precision was assessed by testing 20 replicates of 10 unique extracts (five for intra-assay and five for inter assay) with calprotectin concentrations across the analytical measuring range (15.6 – 500 μg/g). A method comparison was assessed qualitatively and quantitatively by testing 29 extracts on both the QUANTA Lite® Calprotectin ELISA and the PhiCal™ ELISA kit (Eurospital SpA). The reference range (<120 μg/g) was assessed using extracts from healthy donors (n=20). Linearity was assessed by diluting 4 extracts with high calprotectin concentrations (259.5 to 1,453.0 μg/g) until extinction. The limit of blank (LOB) was determined by testing 20 replicates of the assay diluent and using the following equation: Mean of the blank + 1.645 * SD of the blank. The limit of detection (LOD) was determined by testing 20 replicates of one extract with a low calprotectin concentration and using the following equation: LOB + 1.645 * SD of low extract. The limit of quantitation (LOQ) was determined by converting the LOD to μg/g. **Results:** Intra- and inter-assay precision coefficient of variation (CVs) were <9.4% and <10.5%, respectively, for all extracts. The method comparison showed 93.1% qualitative concordance between the two ELISA kits. Quantitative comparison assessed by linear regression showed a slope of 1.21 and R² of 0.987. 100% of the results from the normal donors were less than the positive cutoff, confirming the manufacturer's reference range. Linearity of the assay ranged between 81.8%-104.0% at concentrations from 1,453 μg/g-2.0 μg/g. The LOB was 0.130 optical density (O.D.). The LOD was 0.162 O.D. Conversion of the LOD to μg/g led to an LOQ of 9.8 μg/g. **Conclusion:** The QUANTA Lite Calprotectin ELISA kit is both a robust and reliable test for the quantitation of calprotectin in feces. The test may be useful prior to more invasive procedures (i.e. colonoscopy, endoscopy) used to evaluate patients suspected of IBD.

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Quantitation of Eculizumab in serum by microLC-ESI-Q-TOF MS

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Background: Eculizumab (Soliris, Alexion Pharmaceuticals) is a recombinant humanized therapeutic monoclonal immunoglobulin (mAb) IgG2/4 kappa targeting factor C5 in the terminal pathway of the complement cascade. It is approved for treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. As this is a high cost therapy that is at times life-long, effective therapeutic drug monitoring is good practice for monitoring treatment efficacy.

Objective: The goal of this study was to establish that the intact light chain portion of the mAb could be used to quantify eculizumab in patient serum using microLC-ESI-Q-TOF mass spectrometry.

Methods: Stock eculizumab was obtained from the manufacturer at 9.8mg/mL. Dilutions were made in pooled serum to produce standards and controls. Pooled serum spikes at 10 concentrations between 25 and 1000mcg/mL were evaluated over multiple runs to establish LOD, LOQ, and analytical measuring range. Pooled serum spikes at 3 concentrations where used to simulate quality control and give initial data for intra- and inter-assay precision. A subset of neuromyelitis optica patients receiving eculizumab previously quantitated by ELISA was obtained (N=8). Serum aliquots frozen at -20°C were thawed and 20mcL were treated with 200mcL Melon Gel (Thermo Fischer) to purify immunoglobulins. A volume of 20mcL of supernatant, 10mcL 200mM DTT, and 20mcL 50mM ammonium bicarbonate were placed into a 96-well plate and incubated for 15 minutes; 55°C. A volume of 5mcL reduced sample was injected on an Eksigent microLC system and separated by reverse phase chromatography. Mass spectra were collected on an ABSciex Triple TOF 5600 mass spectrometer. Analyst TFv1.6 was used for instrument control and data review.

Results: The average molecular mass of the light chain amino acid sequence, as provided by Alexion, was calculated to be 23,134.75Da. The +11 charge state of eculizumab, verified from spikes in pooled serum, was used to generate extracted ion chromatograms, instead of the average molecular mass of the deconvoluted mass spectrum. The peak observed from the extracted ion chromatogram was summed, and the area under the +11 charge state peak was manually integrated.

Imprecision was evaluated, using the coefficient of variation, for both intra- (N=12 replicates) and inter-assay (N=8 runs) at 75mcg/mL (4%; 6%), 150mcg/mL (7%; 8%), and 300mcg/mL (13%; 12%). A linear response was observed from 75mcg/mL to 400mcg/mL; R2=0.9957. LOD was defined as 50mcg/mL and LOQ as 75mcg/mL. Interference was evaluated by spiking eculizumab along with three other mAbs into serum. All were chromatographically separated and identified by their unique

+11 charge state mass. Accuracy was determined by comparison to ELISA in patient samples; y=1.0972x-33.857; R2=0.8855. Recovery studies also verified the accuracy of the assay; 104%.

Conclusions: We have demonstrated that microLC-ESI-Q-TOF MS can quantify eculizumab using the response for the +11 charge state of the intact light chain. While more clinical studies of patients taking eculizumab are warranted to better define therapeutic thresholds, this analytical approach has the potential to be quickly adaptable to other drugs in this class and to significantly improve patient care.

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CEREBROSPINAL FLUID FREE KAPPA CHAINS AS A POSSIBLE BIOMARKER TO ASSAY RISK CONVERSION TO MULTIPLE SCLEROSIS

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Background:

Multiple Sclerosis (MS) is the most common demyelinating disease of the central nervous system. In most patients, MS initiates with a clinically isolated syndrome (CIS). A certain number of CIS develop the disease over time, while another group of patients never convert to MS. Therefore, the search for novel biomarkers who predict conversion from CIS to MS is of major importance for early treatment in MS. Aim: Assay the predictive value of free kappa chains (FKC) for the conversion of CIS patients to MS, and compare with other parameters: oligoclonal IgG bands (OCGB), IgG index and magnetic resonance imaging (MRI) criteria used in clinical practice.

Methods:

FKC levels were analyzed in cerebrospinal fluid (CSF) from 176 patients: 41 with normal pressure hydrocephalus (NPH), 77 with CIS, and 29 with relapsing-remitting MS. FKC levels were quantified by nephelometry using the Freelite® Human Kappa Free kit (Binding Site Ltd) on the Siemens® BN II analyzer. SPSSv20 was used for statistical analysis.

Results:

Using a cut-off value of 0.38mg/L determined from the mean CSF-FKC levels of the NPH patients plus 2SD, 45 CIS patients were found above this threshold and 32 below. CIS patients above 0.38 mg/L were found to be at greater risk of conversion to MS than patients with FKC values below this limit (p<0.0001). This predictive value of the FKC is further strengthened by the fact that both CIS groups show statistical significant differences when comparing Barkhof-Tintore criteria, MRI and OCGB criteria. Furthermore, high FLC levels are also strong predictors of positive OCGB and IgG index. Further results for both groups are presented in table 1.

Conclusion:

In our cohort, high CSF-FKC values above 0.38 mg/L increased the risk of conversion to MS. These results suggest CSF-FKC as a possible biomarker to predict conversion to MS.

Table 1: clinical and laboratory data of CIS patients			
	Group 1: patients with CSF-FKC > 0.38 mg/L	Group 2: patients with CSF-FKC < 0.38 mg/L	p value
Patients (n)	45	32	
Gender (females %)	37 (82.2)	23 (71.9)	
Age [mean (CI95%)]	34.6 (31.8-37.3)	36.2 (31.9-40.6)	
Oligoclonal IgG bands in CSF (+/-)	45/0	3/29	<0.0001
IgG index (> 0.5/< 0.5)	45/0	17/15	<0.0001
≥ 3 Barkhof-Tintore criteria (%)	29 (44)	7 (10.6)	0.0040
Fulfilled MRI and OCGB criteria (yes/no)	29/14	3/20	<0.0001
Conversion to MS (%)	51.5	4.6	<0.0001

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Epitopes of human and microbial transglutaminase are similarly recognized by celiac disease sera

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Introduction: The use of microbial transglutaminase (mTg) in the food industry is expanding alongside its ingestion in Western diet. Being a member of the human endogenous tTg, the mTg shares multiple functional similarities. However, immunogenic comparison of the two enzymes in celiac disease (CD) is lacking.

Materials and methods: Complexing mTg and gliadin results in mTg neo-epitope (mTg neo). These complexes were purified by asymmetric field flow field fractionation and confirmed by multi angle light scattering and SDS-PAGE. Sera of 81 CD patients (mean age 30± 17) and 81 healthy controls (mean age 29± 21) were analysed using the following ELISAs: AESKULISA® tTg New generation (tTg-neo-epitopes) IgA and IgG, AESKULISA® Gliadin IgA and IgG, AESKULISA® DGP IgA and IgG and AESKULISA®s against mTg and mTg neo-epitopes (Research use only (RUO) Kits as IgA and IgG).

Results: Purified mTg-neo IgG and IgA (AUC=0.92, 0.93, respectively) showed an increased immunoreactivity compared to single mTg and gliadin (p<0.001) but similar immunoreactivity to the tTg-neo IgG and IgA ELISA (AUC=0.94, 0.95, respectively). Using a competition ELISA, the mTg- and tTg-neo-epitopes have identical outcomes in CD sera both showing a decrease in optical density of 55±6%, (p<0.0002). Sera with high antibody titre [U/ml] against the tTg neo-epitope show also high antibody activities of the mTg neo-epitope and vice versa indicating the presence of similar epitopes within the Tg-gliadin complexes.

Conclusions: mTg and tTg display a comparable immunopotent epitope. mTg neo-epitope IgA and IgG antibodies are immunogenic in CD. If substantiated it will impact the food industry additive policy and regulation

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Anti-neo-epitope tTG complexed to gliadin are more reliable then tTG for celiac disease diagnosis

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Introduction: The guidelines of ESPGHAN for the diagnosis of pediatric celiac disease (PCD) rely on anti-human tissue transglutaminase (tTg) as the prime and unique antibody for screening PCD population. None of the CD-associated antibodies has challenged tTg premiership. tTg complexed to gliadin presents neo-epitopes and antibodies against the complex are called tTg neo-epitope (tTg-neo). Reliability of anti-tTg and tTg-neo antibodies in diagnosis of PCD was compared.

Material and methods; 95 pediatric CD patients (mean 8.3y), 99 normal children (NC) (8.5y) and 79 normal adults (NA) (28y) were tested using the following ELISAs detecting IgA, IgG or both IgA and IgG: AESKULISA® tTg (tTg; RUO) and AESKULISA® tTg New Generation (Neo-epitope tTg complexed to gliadin). The results were compared to the degree of intestinal injury, using revised Marsh criteria.

Results: A significantly higher OD activity was detected for tTg-neo IgA, IgG and IgA+ IgG than for tTg (p<0.0001, p<0.0001, p<0.001, respectively). tTg-neo IgA, IgG correlated better with intestinal damage than tTg (r2=0.968, 0.989 compared to 0.909, 0.488 (p<0.001), respectively). Antibody Sensitivity, Specificity, Positive predicted value, Negative predicted value and AUC for tTG-neo IgA+G were: 95.8, 99, 98.9, 96.1, 0.984, and for tTG IgA+G: 83.2, 100, 100, 86.1, 0.961, and the Significance between the two antibodies performances: p<0.0001 0.0001 0.0001 0.0001 0.0001, respectively. Coclusions: The tTg-neo IgA, IgG and IgA+IgG isotypes exhibited a higher OD activity and better reflected intestinal damage in PCD, compared to tTg isotypes. The tTg-neo combined IgA+IgG ELISA kit had higher sensitivity and a comparable specificity for the diagnosis of childhood CD. tTg neo should be included in the ESPGHAN diagnostic flow chart.

The incidence/prevalence of autoimmune diseases is increasing worldwide

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Introduction: Epidemiological data provide strong evidence of a steady rise in autoimmune disease (AD) throughout westernized societies over the last three decades. Multiple publications exist, describing past or actual incidences/prevalence of individual ADs, however, long term studies on selected populations are scarce.

Aims: to calculate the % increases per year of AD frequencies worldwide and analyze the differential increases of AD per country and disease, and identify geoepidemiological trends.

Methods: A systematic review was performed to identify incidence and prevalence of ADs. Studies from the last 30 years were identified using Medline, Google, and Cochrane Library databases. Only long-term regional or national long-term follow-ups are reported

Results: The means ± s.d. of the increased %/year incidence and prevalence of ADs worldwide were 6.1±4.9 and 11.2±12.8, respectively. Allocating these annual % increases to neurological, endocrinological, rheumatic and gastrointestinal ADs revealed the following trends: 9.65, 6.2, 4.0, and 3.7%, respectively. In all of these, differences between old vs new frequencies were highly significant (p< 0.0001). The diseases showed high to low increased %/year frequencies: myasthenia gravis, celiac disease, SLE, IDDM and IBD, with 17.4, 12.2, 3.7, 2.6 and 2.4%, respectively. Geoepidemiologically, the following countries had high to low %/year ADs frequency increases: Japan, Italy, Brazil, Denmark, Norway, Sweden, UK, Israel, USA, Canada, Finland and Serbia with 23, 20, 12.4, 10.6, 10.3, 9.8, 9, 5.6, 5, 3.3, 3.1 and 0.8%, respectively.

Conclusions: Despite multiple reports on ADs frequencies, long-term longitudinal follow-ups are scarce. Reviewing available literature, it can be deduced that incidences and prevalences of ADs have increased significantly over the last 30 years. Neurological (MS), endocrinological (IDDM) and gastrointestinal (CD, IBD) ADs and Japan, Italy, Brazil and Denmark increased most. These observations point to a stronger influence of environmental factors as opposed to genetic factors on AD development.

Determination of Deamidated Gliadin Peptide Antibodies in Dried Blood Spots

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Background: With increasing prevalence and heightened awareness of celiac disease, consumers and professionals are seeking a convenient, precise, and accurate method for celiac serologic testing. The differential diagnosis for celiac disease includes the analysis of blood for the detection and quantitation of antibodies to deamidated gliadin peptide (DGP). Our objective was to develop and validate DGP antibody testing in dried blood spot (DBS) samples. Single-use, self-retracting bloodletting devices enable the self-collection of capillary blood from lay users. Highly-standardized filter paper collection and transport media are increasingly used for clinical laboratory testing. The employment of sensitive chemiluminescent immunoassay testing facilitates expansion of DBS offerings in clinical laboratories for the reliable analysis of specific proteins, including DGP immunoglobulin A (DGP-IgA) and DGP immunoglobulin G (DGP-IgG).

Methods: Capillary blood samples are collected using SurgiLance™ sterile lancets, spotted onto PerkinElmer® 226 Spot Saver Cards, and permitted to dry. Cards are desiccant packaged and shipped via standard postage to the laboratory. From a 50µL blood drop, four 3-millimeter spots are punched using a calibrated hole punch and extracted into a buffered solution, equivalent to the established on-board sample dilution of the analyzer. Extracts are analyzed on an INOVA Bio-Flash® chemiluminescent analyzer in microsampling mode, bypassing further on-board dilution. Results are read from 5-point DBS calibration curves, derived from high-titer standards of DGP-IgA and DGP-IgG obtained from INOVA. Analytical precision, linearity, recovery, accuracy, and stability of DGP-IgA and DGP-IgG were assessed.

Results: For DGP-IgA, intra-assay and inter-assay precision coefficients of variation (CV) (n=20) at 289.0 concentration units (CU) were 1.4% and 3.2%, and at 34.8 CU were 3.0% and 6.1%, respectively. For DGP-IgG, the intra-assay and inter-assay CV (n=20) at 596.0 CU were 0.9% and 3.8%, and at 11.6 CU were 2.0% and 8.2%,

respectively. DGP-IgA was confirmed linear between 5.2 - 1144 CU, with recovery of expected units ranging from 96.4% - 100.8% (n=6). DGP-IgG was confirmed linear between 2.8 - 1646 CU, with recovery ranging from 95.1% - 101.9% (n=6). Known celiac-positive donors provided samples to permit sample matrix comparison. Least-squares regression analysis comparing DGP-IgA values in serum to DBS (n=44, range 5.2 - 762.0 CU) yielded a correlation coefficient of 0.969, y = 1.13x + 7.77; standard error of estimate ($S_{y/x}$) = 44.9. Least-squares regression analysis comparing DGP-IgG values in serum to DBS (n=44, range 3.0 - 769.0 CU) yielded a correlation coefficient of 0.978, y = 1.05x - 4.21; $S_{y/x}$ = 33.0. Both DGP-IgA and DGP-IgG demonstrated 30-day stability with collection cards stored desiccated in sealed Ziploc™ bags at 25°C or lower.

Conclusion: The analytical method developed and validated for DBS DGP-IgA and DGP-IgG testing on the INOVA Bio-Flash provides a precise and accurate means of determining serologic status for celiac disease. The collection system for this method has proven to be well-accepted by lay users, with 99% of submitted samples of adequate quantity and quality. The transportation system provides extended stability to preserve sample integrity, enabling shipping from remote locations to a central laboratory for analysis.

Can Serum Light Chain Tests Replace Diagnostic Tests for Urine Free Light Chains? Positive Urine Free light Chains but Negative Serum Free Light Chain levels in two cases

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Background: Serum testing including SPE, IFE and free light chains [FLCs] are considered the important tests in diagnosis and follow-up of plasma cell dyscrasias. Among these serum FLCs provide more sensitive alternative to 24 hour urine testing in the diagnosis and monitoring of light chain MM. In contrast urine K/L ratios are not as dependable and UPE, UIFE and Urine free light chains analysis are increasingly considered as wasteful and therefore expendable. In our laboratory we perform the full gamut of serum and urine testing on myeloma patients for diagnosis and follow-up

Methods: We report two cases, both had initial diagnosis of monoclonal gammopathy of uncertain significance [MGUS] or light chain disease and were monitored with serum and urine free light chains as well as with SPE and IFE. Free light chains were [measured by immunoassay on Beckman Image platform]. In both cases serum light chains and ratio were normal and the urine free light chain assays were diagnostic emphasizing the importance of urine testing in these cases.

Results: The first case is of a 60 year old gentleman, who presents with a kappa light chain disease. He had initially presented in 2010 with an MGUS (IGMK) with normal albumin, calcium, CBC. He now has a normal SPEP and normal serum FLC [K 12.1 and L 11.9mg/L; K/L ratio 1.02]. Urine IFE shows an atypical restricted band with urine FK 63 mg/L and FL 1.6 mg/L and K/L ratio of 40. The patient had a complex cystic kidney disease with unremarkable serum albumin, creatinine, calcium, CBC, eGFR.

The second case is a 58 year old gentleman with a diagnosis of Multiple myeloma [IgG Lambda]being treated with chemo and radiation. He has anemia but normal chemistries. An atypical restriction is seen on serum SPE and IFE showed IgG Lambda and an additional band in Kappa but the serum FLC are within normal limits [S-FK <2.9 and S-FL 6.6 mg/L; K/L ratio <0.44]. The urine free light chains are abnormal (U FK 61.8 and UFL 3.2 mg/L and a high K/L ratio of 19.31.

Conclusion: In both cases serum free light chains and ratios were normal and the urine free light chain assays were diagnostic emphasizing the importance of urine testing in these cases. Why these patients repeatedly fail to show serum free light chains is not clear and requires further investigation.

Alternatives to Oligoclonal Banding Electrophoresis in CSF: Method Comparison with Quantitative Free Light Chains and Accurate Molecular Mass Measurement of Immunoglobulins

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Background: Isoelectric focusing coupled with IgG specific immunoblotting (IgG-IEF) is routinely used to identify immunoglobulins specific to the CNS compartment as part of the diagnostic criteria for multiple sclerosis (MS); i.e. oligoclonal banding (OCB). However, it is a labor-intensive technique with subjective interpretation of IgG bands from paired cerebrospinal fluid (CSF) and serum. Measurement

of the concentration of free light chains (FLC) in CSF by nephelometry has been reported as an alternative measurement to support the diagnosis of MS. In addition, microLC-ESI-Q-TOF mass spectrometry can be used to identify both monoclonal and polyclonal immunoglobulins using accurate molecular mass. We compared the diagnostic performance of the IgG-IEF reference method with FLC by nephelometry and microLC-ESI-Q-TOF mass spectrometry to identify immunoglobulins in CSF.

Methods: Forty-four residual paired CSF/serum samples previously analyzed as positive OCB (OCB$^+$, N= 25) and negative (OCB^{neg}, N=19) by IgG-IEF (Helena SPIFE 3000) were used for this study. FLC kappa and lambda were measured by nephelometry (The Binding Site) in serum and CSF. Serum immunoglobulins were purified using Melon Gel (Thermo Fischer). Samples were reduced with dithiothreitol then analyzed by microLC-ESI-Q-TOF MS on an AB SCIEX Triple TOF 5600 mass spectrometer. Clones unique to CSF and serum were identified using accurate molecular mass (monoclonal immunoglobulin Rapid Accurate Mass Measurement (miRAMM)). Readers were blinded to OCB results.

Results: The mean±SD number of IgG bands observed by IEF was 9.2±3.6 for OCB$^+$ samples, whereas in the OCB^{neg} cohort it was 0.2±0.4. Concentrations of kappa and lambda were ~12-fold and ~6-fold higher in OCB$^+$, respectively ($p<0.0001$). Receiver Operating Characteristic (ROC) curve analysis showed an AUC of 0.976 for kappa FLC concentration in CSF, and a cut-off ≥0.0623 mg/dL provides a sensitivity of 100% with specificity of 83% in comparison to IEF. Analysis of the sum of FLC in CSF provided similar results (AUC 0.970) when a cut-off ≥0.1200 mg/dL is applied. Concentrations of FLC in serum did not correlate with OCB results (p>0.05). MicroLC-ESI-Q-TOF oligoclonal profiles were in 100% agreement with IEF. In the OCB^{neg} cohort, CSF did not contain any light chain clones in 16 samples (84%). 3 samples had clones whose accurate mass (m/z ratio) matched in both serum and CSF and therefore were interpreted as negatives. For OCB$^+$, paired CSF/serum analysis showed that 4 samples had unique clones in CSF, none detected in serum. 21 CSF samples had clones in both serum and CSF, however in CSF there were additional unique clones whose accurate masses were not identified in serum, and reported as positives.

Conclusion: FLC measurement in CSF by nephelometry shows excellent correlation with IEF with the benefit of potentially eliminating the need of a paired serum for interpretation. MicroLC-ESI-Q-TOF had equivalent performance to IEF to measure immunoglobulins light chains in CSF, with the advantage of being automated and allowing for unambiguous identification of the accurate mass of the clones produced intrathecally.

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Cryoprecipitate as a Quality Control Material for Cryoglobulin Analysis

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Background: Cryoglobulins are serum immunoglobulins that precipitate at a temperature less than 37^{0}C. The presence of cryoglobulins in vivo are associated with serum hyperviscosity and a variety of clinical symptoms including, but not limited to, purpura, arthralgias, myalgias, weakness, Raynaud's phenomenon, visual disturbances, headache, vertigo, loss of consciousness and stroke. These clinical symptoms are not specific to the presence of cryoglobulinemia; thus accurate detection of cryoglobulinemia is important. Cryoglobulin analysis involves incubation of separate aliquots of patient serum at 4°C and 37°C for up to 72 hours. Cryoglobulins, if present, will precipitate at 4°C while the aliquot at 37°C should remain clear. A challenge of cryoglobulin analysis is the lack of suitable commercially available quality control materials. In our search for a material that would reliably precipitate at 4°C but remain clear at 37°C we investigated whether expired or returned cryoprecipitate from the hospital blood bank would serve as a suitable positive QC material. Per protocol, cryoprecipitate returned to the blood bank is discarded as it expires 6 hours after thawing. **Objective:** The objective of our study was to determine if expired or returned cryoprecipitate from the blood bank could serve as a positive control material for cryoglobulinemia analysis.

Methods: Returned cryoprecipitate was quarantined and stored at 4°C until retrieved by chemistry laboratory staff. Thawed cryoprecipitate bags were pooled and frozen as 2.5 mL aliquots at 20°C until use. Validation of the cryoprecipitate for use as control material consisted of incubating separate thawed aliquots of cryoprecipitate on 5 non-consecutive days in Wintrobe tubes at 4°C and 37°C for 72 hours. At the end of the 72 hour incubation period the Wintrobe tube held at 4°C was centrifuged in a refrigerated (4°C) centrifuge at 590 RCF for 30 minutes after which cryocrit was assessed visually.

Results: Analysis of the 5 cryoprecipitate aliquots in Wintrobe tubes after 72 hours of 4°C incubation consistently demonstrated a white flocculent material visually

consistent with the presentation of a cryoglobulin. The white flocculent material re-dissolved when warmed to 37°C and separate aliquots of cryoprecipitate incubated at 37°C remained clear; indicating that the flocculation observed at 4°C was a satisfactory surrogate for the cryoglobulin precipitation phenomenon. Centrifugation of the flocculent material revealed that four of five aliquots had a reproducible cryocrit of 10%. The fifth aliquot had a cryocrit of 6%. Investigation of the lower cryocrit value in this one aliquot revealed that the fifth aliquot was thawed in a 37°C water bath as opposed to being thawed at room temperature. This difference in procedure revealed that the thawing process of the cryoprecipitate can contribute to the variability in the QC cryocrit value.

Conclusion: Cryoprecipitate unused for patient care can be repurposed as an assay control for the detection of cryoglobulinemia. Addition of a material that reliably precipitates at 4°C and re-dissolves at 37°C replicates the analytical procedures needed for assessment of cryoglobulinemia and ensures appropriate sample handling of the cryoglobulin tubes during the extended 72 hour incubation and analysis time.

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Development and Validation of an Innovative Allergen-specific IgE Assay on the HOB Lumiray®1260 Automated Chemiluminescence Immunoassay Analyzer

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Background:

Besides the skin tests, in vitro determination of allergen-specific IgE (sIgE) represents an important approach to the diagnosis and treatment of allergy. Recently, the innovative HOB Allergy testing, coupling with the fully automated, random-access Lumiray®1260 chemiluminescence immunoassay system has been launched. The HOB Allergy testing is based on the nanoparticle magnetic properties and chemiluminescence technology by using the indirect method. The magnetic streptavidin coated nanoparticles enable the separation and washing procedures; the bounded sIgE reacts with selected biotin-labeled allergen and is detected by the anti human IgE conjugated alkaline phosphatase triggers the chemiluminescence substrate for quantitative determination of IgE antibodies in serum.

Methods:

In this study, the HOB allergy assay was evaluated and compared to the ImmunoCAP (Phadia AB, Sweden) according to the CLSI guidelines. The analytical performance of ten allergens was compared. It covered five inhalant allergens including D1 (*Dermatophagoides pteronyssinus*), D2 (*Dermatophagoides farina*), E1 (cat epithelium), E5 (dog dander) and M6 (*Alternaria alternate*) and five food allergens including F3 (codfish), F4 (wheat), F13 (peanut), F23 (crab) and F24 (shrimp).

Results:

The HOB Allergy sIgE assay performed excellent linearity in the assay ranging from 0.1- 100 kU/L and high precision (the within-run CV% were <5.1%, the between-run CV% were <7.4%, and the total precision CV% were <9.8%). The limit of detection was < 0.01 kU/L. Bilirubin (up to 50 mg/dL), hemoglobin (up to 400 mg/dL), and lipid (up to 2000 mg/dL) did not affect the IgE quantitative detection in serum. It showed no cross-reactivity with other human classes of immunoglobulins (IgG, IgD, IgA and IgM) at physiological concentrations. Total of 1547 clinical samples were compared between HOB Allergy assay and Phadia ImmunoCAP at the cut-off of 0.35 kU/L for the ten allergens, the sensitivity concordance were ranged from 76.2% (F3) to 97.3% (E1), the specificity concordance were ranged from 92.0% (D1) to 100% (F4), the total agreement were ranged from 87.4% (D1) to 96.3% (F13).

Conclusion:

The HOB Allergy system is an innovative allergen-specific IgE assay performing a linear reaction, an extended working range, good precision and reproducibility on Lumiray® 1260. Excellent agreements are observed between HOB Allergy and ImmunoCAP for the detection of specific IgE. The HOB Allergy system with automated, random access and multiprofiling capabilities provides the fast and accurate detection of sIgE that leads to greatly improve the diagnosis and treatment of allergy patients.

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SLE-KeyTM Rule-Out Serologic Test for Excluding the Diagnosis of SLE

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Background:Systemic Lupus Erythematosus is associated with a large spectrum of autoantibodies, but currently there is no reliable serologic diagnosis. Each autoantibody individually fails to discriminate with sufficient specificity and/or sensitivity SLE patients from healthy controls or from subjects afflicted with other autoimmune diseases. Currently a diagnosis of SLE is based on multiple criteria and can take years of monitoring.

Methods: We developed the previously described iCHIP™1 as an effective SLE rule-out diagnostic test by profiling with an antigen microarray multiple, distinct autoantibody reactivities in the sera of SLE patients compared to healthy controls followed by informatics analysis to rule out a diagnosis of SLE. An initial set of 200 antigens associated with SLE was selected from the literature bolstered with sets of proprietary markers developed by ImmunArray. In addition, we analyzed a subset of ANA(+) samples from the Healthy Control pool. We collected serum samples from 250 SLE patients from four independent sources [Albert Einstein College of Medicine, Medical University of South Carolina, Johns Hopkins University and Emory University] and compared them with age race and gender matched sera of 250 healthy control samples independently sourced. We tested these samples using the ImmunArray SLE-Key™ - a proprietary microarray that displays multiple antigens representing a range of SLE-associated biochemical pathways. The SLE-KeyTM chip is printed using a SciFLEXARRAYER SX (Scienion AG, Berlin Germany. After processing, chips were scanned on a G2565 Series C Microarray Scanner (Agilent Technologies, Santa Clara, CA). Multiple classification algorithms were used to develop a set of classifiers. Training and verification was performed on a subset of 200 SLE patients and 200 healthy controls using 4 independent classification methods (Support Vector Machine (SVM), Logistic Regression (LR), Quadratic Discriminant Analysis (QDA), Linear Discriminant Analysis (LDA)). Validation was performed on an additional set of 50 SLE patients and 50 healthy control samples. For the ANA(+) subset of Healthy Control samples, 26 tested positive (>1:40 dilution; ANA IFA Screen, Test Code 249, Quest Diagnostics, Madison, NJ) out of 136 (19.1%) samples with sufficient residual volume for testing. These positive samples were also analyzed on the SLE-Key TM chip.

Results: The SLE-Key TM classifer successfully differentiated SLE patients from healthy subjects with a sensitivity of greater than 90% and specificity of greater than 70%. For the ANA(+) healthy control samples, the SLE-KeyTM classified 50-80% of these samples as healthy control depending on classification method chosen.

Conclusion: The SLE-Key TM multiplex test can be used to assist physicians in ruling out serologically a diagnosis of SLE with a sensitivity of >90%. The SLE-Key TM may also show utility in evaluating ANA(+) otherwise healthy patients after further confirmatory studies are performed. Work comparing the testing performance of the SLE-Key TM in direct comparison to standard serologic testing in SLE patients is ongoing.

1 Fattal, I, et al; Immunology 2010, 130, 337-343

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Comparison of NK Vue, quantitative IFNγ ELISA, with flowcytometric NK activity test

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Backgrounds: Natural killer (NK) cells play a key role in innate immune responses. NK cell activity is generally measured by flowcytometric CD107a degranulation assay or ^{51}Cr release assay. Conventional NK activity measurement methods require complex experimental setups, such as peripheral blood mononuclear cells (PBMC) purification, NK cells stimulation with K562 cells or cytokines, and professional interpretation of flowcytometric analysis or handling with radioactive materials. In this study, we evaluated analytical performances of NK Vue (ATgen, Sungnam, Korea), which detect NK cell activity by measuring the secretion of IFNγ from *ex vivo* stimulated NK cells in whole blood using ELISA.

Methods: NK Vue assay was done using the supernatant from whole blood following incubation with PROMOCA (ATgen), a bioengineered recombinant cytokine that activates NK cells, for 24 hours in a 37°C CO_2 chamber. Precision and linearity studies

of NK Vue were conducted according to Clinical and Laboratory Standards Institute guidelines. For the comparison of NK Vue with flowcytometry, 83 participants were enrolled with informed consent to study. For flowcytometry, PBMC separated from the matched heparinized blood were incubated with K562 cell line for 6 hours and stained with CD45-FITC, CD3-ECD, CD16/CD56-PC7, CD107a-PC5, and cytoplasmic IFNγ-PE. CD107a expression and intracytoplasmic IFNγ level in CD56^{-}CD16^{+} NK cells as parameters of NK cell activity were determined by flowcytometry.

Results: Within laboratory precision of NK Vue for low, middle and high level was 7.5%, 9.1% and 6.3% CV, respectively. The measurement range of NK Vue from 35.5 pg/mL to 884.6 pg/mL showed a clinically relevant linearity ($R^2 = 0.9987$). Secretory IFNγ measured by NK Vue showed good correlation with CD107a expression determined by flowcytometry (Pearson correlation coefficient 0.413, P<0.001), especially in a group of IFNγ ≥100 pg/mL (Pearson correlation coefficient 0.759, P<0.001). However, secretory IFNγ by NK Vue did not correlated with cytoplasmic IFNγ by flowcytometry.

Conclusions: The precision of NK Vue was less than 10%, and linearity confirmed over a range of 35.5 pg/mL to 884.6 pg/mL. The significant correlation of secreted IFNγ by NK Vue with CD107a expression by flowcytometry was noted. The findings in this study suggest that NK Vue assay, less laborious and more compactible than traditional NK cell activity measurement, could be a feasible method to estimate and monitor the NK cell function in clinical setting.

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Profiling Sialylation Status during Monocytes Differentiation into Macrophages

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Background: Sialic acids (SAs), a family of 9-carbon containing acidic monosaccharides, often terminate the glycan structures of cell surfaces and are involved in many biological functions including early fetal development, cellular recognition and adhesion, and its utilization by microbes. While it is clear that cell surface SAs are highly involved in the immune system, the sialylation status related to individual immune cells and their activation state and functions are still unknown. In this study, we combined a newly developed LC-MS/MS method, along with flow cytometry and confocal microscopy to profile the changing pattern of SAs during monocytes differentiation and polarization. This is the first report of applying LC-MS/MS to the determination of SAs in monocytes and macrophages. This work will lead to a better understanding of the physiological and pathological roles of SAs in the immune system.

Objective: To study the level and pattern change of SAs during monocytes differentiation and polarization with LC-MS/MS, flow cytometry and confocal microscopy.

Methods: THP-1 monocytes were used as a model immune cell. The differentiation of monocytes to macrophages was accomplished by PMA. The further polarization to classically activated M1 macrophages and alternatively activated M2 macrophages was achieved by addition of LPS and IFN-γ, IL4 and IL13, respectively. A newly developed LC-MS/MS method was employed to quantify free SA in the culture medium and cellular SA in the cell lysate. Flow cytometry was optimized to quantify α-2,3 and α-2,6 linked SAs on the cell surfaces with MAA and SNA lectin labeling, respectively. Confocal microscopy was performed to visualize SAs on the cell surfaces and inside the cells. Sialidase activity was measured to confirm the change in SA amounts in the culture medium.

Results: After PMA treatment, free SA in the culture medium increased from 4.18±0.01 ng/ml to 11.57±0.78 ng/mL, α-2,3 SAs on the cell surface decreased 35%, and α-2,6 SAs decreased 25%. These results were confirmed by sialidase activity assay, which showed the activity of major sialidase (Neu1) increased by more than a factor of 2. Cellular SAs increased from 718±6 ng/mL to $(1.59\pm0.5)\times10^3$ ng/mL. This change was verified by confocal microscopy, which showed the increase of both α-2,3 and α-2,6 SAs inside the cells. Moreover, after M1 and M2 polarization, cellular SA decreased 26% in M1 macrophages and increased 13% in M2 macrophages compared with only PMA treatment.

Conclusions: When THP-1 monocytes differentiate into macrophages, both the level and pattern of sialylation changes. The increase of free SA in the medium and decrease of α-2,3 and α-2,6 SAs on the cell surface indicate the elevation of the sialidase activity. The increase of cellular SAs is confirmed by both LC-MS/MS and confocal microscopy. However, the SA change in M1 and M2 polarization can only be determined by LC-MS/MS owing to the technique's sensitivity and accuracy. Overall, this study provides for the first time a global investigation of the cellular sialylation status of monocytes and differentiated and polarized macrophages. It has potential

significance in understanding the pathology and diagnosis of disorders involving monocytes and macrophages.

Circulating BCMA Binding to Its Ligand BAFF Prevents Normal Antibody Production in Multiple Myeloma Patients

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Background:

A hallmark of multiple myeloma (MM) is the low levels of uninvolved immunoglobulin (Ig) levels. B-cell maturation antigen (BCMA) is a receptor expressed in mature non-malignant and malignant B lymphocytes, including plasma cells. We previously demonstrated that BCMA is present in the serum of MM patients (pts) and that its levels predict overall survival (Sanchez et al. Br J Haematol 2012). We hypothesized that circulating BCMA binds to its ligands, preventing normal plasma cell development and antibody production in MM patients.

Methods:

BCMA-Fc and control Ig were obtained (R&D Systems). Human BCMA and mouse BAFF, and mouse plasma IgA, IgA and IgM levels were measured with ELISA (R&D Systems & Bethyl Laboratories). rhBCMA-mBAFF complexes were determined using an ELISA. Plates were pre-coated with a monoclonal mouse antiBAFF capture Ab. Plasma samples were incubated and an antihuman-BCMA detection Ab was added. Human serum IgA and IgG levels were determined in MM patients using nephelometry (Immage 800, Beckman Coulter). Hevylite® Assays (Binding Site) were used to quantify the levels of heavy-light chain isoform pairs.

Results:

To determine the effects of human BCMA on plasma Ig levels in immune competent mice, rhBCMA-Fc or control Ig-Fc (100 μg) was injected into C57 Bl or Balb/c mice. rhBCMA-Fc resulted in significant decreases in IgA, IgG and IgM levels. Decreases in IgA levels were observed when compared to baseline levels on days 4 and 6 (P = 0.0031 and P = 0.0064, respectively), and the controls (P = 0.0087 and P = 0.0221). Mouse IgG levels also showed a reduction compared to baseline (P = 0.0023), the Ig-Fc (P = 0.0014) and control (P = 0.0129) groups. IgM levels showed similar decreases when compared to the untreated (P = 0.0001) and IgFc (P = 0.0088) groups. We determined if rhBCMA-mBAFF complexes formed *in vivo*. Complexes were detected by ELISA at high levels in plasma from mice dosed with BCMAFc, whereas none were found in samples in control IgFc or untreated mice. Next, we determined the relationship of serum BCMA levels to uninvolved Ig levels in MM pts. For pts with IgA (n = 134) or IgG (n = 313) MM, higher BCMA levels ($\geq$ 100 ng/ml) correlated with below normal levels of uninvolved IgG in IgA MM and uninvolved IgA in IgG MM, whereas lower BCMA levels (< 100 ng/ml) correlated with normal uninvolved levels (P < 0.0001). Using the Hevylite Assay, similar results were observed BCMA levels compared to uninvolved IgG isoforms in both pts with involved IgG lambda (n = 62, P = 0.0006) and IgG kappa (n = 117, P < 0.0001) MM.

Conclusion:

We demonstrate the formation of circulating BCMA-BAFF complexes in MM, and administration of recombinant BCMA to normal mice results in marked reductions in their antibody levels. We also show that BCMA levels inversely correlate with uninvolved Ig levels in MM pts. Thus, the lack of normal antibody production in MM pts results in part from circulating BCMA binding its ligands, preventing production of normal antibody-producing cells.

Comparison between singlex ELISAs and a microfluidics-based multiplex cytokine assay

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Recently, technologies have enabled multiplexing of cytokine immunoassays with substantially less sample volume requirement than the conventional 96-well plate-based single measurand ELISA (singlex ELISA). The study objective was to compare the analytical performance of CyPlex, a microfluidics-based multiplex immunoassay, to singlex ELISAs on measurement of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-α (TNF-α).

68 serum samples were assayed by singlex ELISAs (R&D systems, Minneapolis, MN) and by multiplexed assay (CyPlex by CyVek, Wallingford, CT). Manufacturer-provided calibrators (ELISA) or lot-specific pre-calibration (CyPlex) was used. IL-1β and IL-6 assay antibodies in both methods were from the same supplier. Singlex ELISAs required 600 μL of serum (100 μL each for IL-1β and IL-6, 200 μL each for IL-10 and TNF-α), whereas Cyplex required 25 μL for four cytokines total. The singlex ELISA accommodated 80 samples per plate, excluding blanks, calibrators and quality controls (QCs), and each plate took 4-6 hours to run; Cyplex allowed for 16 samples per cartridge, and each cartridge took 1.5 hours to run (80 samples for 7.5 hours). Table 1 listed assay characteristics including coefficient of variation (CV) and analytical measurement range (AMR). The limited AMR is more of a concern for cytokines with low serum concentrations (IL-1β and IL-10). Assays using the same antibody supply in the two methods showed good linear regression: R^2 = 0.98 for IL-1β, 0.98 for IL-6. Assays with different antibody sources showed poor correlation: R^2 = 0.02 for IL-10, 0.37 for TNF-α. We concluded that the microfluidics-based CyPlex multiplexed immunoassay substantially conserved sample volume than traditional singlex ELISAs for measurement of IL-1β, IL-6, IL-10, and TNF-α (25 μL versus 600 μL). When the same antibodies were used, the two methods showed comparable performance on precisions and reportable ranges, and good correlation between each other.

Table 1. Assay characteristics of the singlex ELISA and Cyplex.						
	Inter-assay CV (number of QCs)		AMR (pg/mL)		Number of samples outside AMR (%)	
Cytokine	ELISA	Cyplex	ELISA	Cyplex	ELISA	Cyplex
IL-1β	4.1 - 8.4% * (20)	8.0% (6)	0.125-8	0.21-2000	28/43 (65%)	45/66 (68%)
IL-6	9% (30)	6.2% (6)	0.156-10	0.52-2000	8/65 (12%)	2/66 (3%)
IL-10	14.5% (24)	9.1% (5)	0.195-50	0.21-2000	32/64 (50%)	0/66 (0%)
TNF-α	6.6% (23)	6.2% (6)	0.50-32	1.31-5000	9/43 (21%)	3/66 (5%)

* The inter-assay precision of the IL-1β singlex ELISA was provided by the manufacturer and was not verified by our lab due to limited reagents.

Analytical Evaluation of The Helena SPIFE 3000 Electrophoresis/Immunofixation System

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Background: Protein Gel electrophoresis (PEP) and immunofixation (IFE) are commonly used in clinical laboratories for the diagnosis and management of patients with monoclonal gammopathies. There are two semi-automated agarose gel electrophoresis systems for PEP/IFE in routine clinical use_the Sebia Hydrasys (Norcross, GA) and Helena SPIFE (Beaumont, TX). The Helena SPIFE 3000 system has the ability to run more PEP (60 vs 30) and IFE samples (15 vs 9) than the Sebia Hydrasys system. Therefore, SPIFE 3000 may potentially allow us to handle the rapidly increasing PEP/IFE test volumes in our institution with an acceptable turn-around-time for our clinicians. The aim of this study was to assess analytical performance of the SPIFE 3000 and compare its performance with the Hydrasys system.

Methods: The evaluation included precision studies, linearity analysis, protein fraction and immunofixation comparison studies. One normal and one abnormal serum sample with hypergammaglobulinemia were used to assess precision for each protein fraction (albumin, alpha-1, alpha-2, beta-1, beta-2, gamma). One serum sample with paraprotein was serially diluted (1:2; 1:4; 1:8; 1:16) and analysed in triplicates to evaluate the linearity for paraprotein quantification in gamma region. 36 serum and 35 urine samples were used for the immunofixation comparison study. The quantification of protein fractions and paraproteins obtained with both Hydrasys and SPIFE 3000 were performed by experienced laboratory technologists.

Result: The total precision of SPIFE 3000 on all protein fractions was between 2.0% and 5.6% CV; the linearity was excellent up to an paraprotein value of 5.1 g/dL (r^2 = 0.997). The mean bias of SPIFE 3000 versus Hydrasys was -0.650 g/dL for albumin, 0.095 g/dL for alpha-1, 0.069 g/dL for alpha-2, 0.344 g/dL for beta, and 0.143 g/dL for gamma fraction, respectively. Immunofixation comparison showed discrepant results in 17% of all samples tested (n=71). SPIFE 3000 accurately characterized all 18 IgG, 6 IgA, and 1 IgM monoclonal bands in serum and 5 IgG, 1 IgA, and 9 kappa monoclonal bands in urine, respectively. However, missing monoclonal bands were seen in: 1/10 kappa, 6/18 lambda in serum; and 2/8 lambdas in urine using SPIFE

3000. Missing bands were also observed with Sebia system: 1/1 IgM, 1/10 kappa, and 1/18 lambda in serum; 3/5 IgG, 1/8 kappa and 1/7 lambda in urine.

Conclusions: For routine protein electrophoresis, the results of our evaluation showed that Helena SPIFE 3000 is a suitable alternative method. Differences in protein fractions were found between two systems; therefore reference intervals should be established for each system. For immunofixation, our study showed that SPIFE 3000 is prone to miss some monoclonal free lambdas. All missing lambda bands observed in this study were coupled with another monoclonal band (i.e. monoclonal IgG lambda, IgG kappa, free lambda) in the serum sample. This could be due to the specificity of the antisera against lambda used in SPIFE 3000 reagent, however, a specific antisera reagent against free light chains (kappa and lambda) provided by Helena did not resolve this problem for the samples tested. Further study is ongoing to understand and resolve this issue.

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Diagnostic Utility of Biomarkers of Systemic Mastocytosis

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Background: Systemic mastocytosis (SM) is a myeloproliferative neoplasm resulting in the accumulation of clonally derived mast cells. When the mast cells are triggered, the released cellular contents cause a variety of symptoms including, flushing, tachycardia, gastrointestinal distress, or loss of consciousness. Current markers of SM include the mast cell secretory molecules tryptase, N-methylhistamine (NMH), 2,3-dinor-11-beta prostaglandin F2α, (2,3-BPG) and leukotriene E4 (LTE4). Patients often present after taking over-the-counter anti-histamines and cyclooxygenase inhibitors. These medications may influence biomarker concentrations.

Objective: To assess the diagnostic utility of all SM biomarkers and assess the influence of medication on analyte concentrations.

Methods: This study was approved by the Institutional Review Board. Urine samples from 169 patients, with a clinically ordered NMH and tryptase were collected. NMH, 2,3-BPG and LTE4 were measured in urine by LC-MS/MS (AB Sciex API 5000). Concentrations were normalized to creatinine (enzymatic method, Roche). SM diagnoses were confirmed by an allergist according to blinded chart review. Clinical sensitivity and specificity for SM diagnosis was determined using cutoffs previously established (tryptase >20ng/mL, LTE4 >104 pg/mg creatinine, NMH >200 ng/mg creatinine, and 2,3-BPG >1382 pg/mg creatinine).

Results: All analytes were significantly higher in patients with SM (Table 1). Tryptase and NMH were not significantly altered by medication status. LTE4 was significantly different in patients taking aspirin while 2,3-BPG was significantly different in patients taking Benadryl. The diagnostic sensitivity (92%) and specificity (60%) of the three urinary markers together was not significantly improved by addition of tryptase (92% sens; 61% spec).

Conclusion: Urinary markers individually or in combination aid in the detection of SM. However, adding serum tryptase to all three urinary markers does not increase performance. Medications could alter the concentrations of urinary markers; however, the effect on diagnostic performance for SM remains to be elucidated.

Table 1: Sensitivity and Specificity for Systemic Mastocytosis Detection Markers

	Systemic Mastocytosis			Aspirin		Benadryl	
	Yes (n=26)	No (n=143)		No (n=124)	Yes (n=45)	No (n=87)	Yes (n=82)
	Mean		Sensitivity,% Specificity, %	Mean		Mean	
Tryptase, ng/mL	55	7	65 96	13	20	14	15
p-value		<0.0001			0.102		0.582
NMH, ng/mg cr.	840	54	77 92	193	287	231	203
p-value		<0.0001			0.080		0.686
2,3-BPG, pg/mg cr.	1517	860	46 84	955	977	852	1076
p-value		<0.0001			0.433		0.024
LTE4, pg/mg cr.	203	109	42 81	103	179	132	114
p-value		0.016			0.017		0.713

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Study of bronchoalveolar lavage flow cytometry analysis in the diagnosis of patients with diffuse interstitial lung diseases

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Background:

Diffuse interstitial lung diseases are a heterogeneous group of respiratory diseases with difficult diagnosis. The study of bronchoalveolar lavage (BAL) by flow cytometry may define typical patterns in different diseases, providing some help in the differential diagnosis. The aim of this study has been to retrospectively analyze the clinical usefulness of flow cytometry analysis of the cellular and lymphocyte subpopulations

Methods:

We have study 104 patients retrospectively. Subjects were diagnosed of interstitial lung disease during 2.5 years. BAL cellular analysis by flow cytometry, histology and CT scan were analysed in different disease subgroup. Percentage of T cells, B cells, NK cells, CD4, CD8 and CD4/CD8 ratio were analysed by flow cytometry as CD3+, CD19+, CD4+, CD8+, CD3+CD4−CD8−, and CD3-CD16+56+.

Results:

The low incidence of diffuse interstitial lung disease in our area was confirmed (1/10000 per year). Idiopathic pulmonary fibrosis (30%) and sarcoidosis (16%) were the most frequent diagnosis. Lymphocytosis and the CD4:CD8 ratio was the most useful parameter. Thus, this ratio was high in sarcoidosis, and it was inverted in extrinsic allergic alveolitis. It was also low in the bronchiolitis obliterans organizing pneumonia. Neutrophils were the predominant population in idiopathic pulmonary fibrosis

Conclusion:

The study of BAL is helpful to discriminate between interstitial lung diseases

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Application of Bioenergetic health index (BHI) in Healthy individuals and Chronic Kidney Disease

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Background: Mitochondria play a critical role in maintaining cellular homeostasis by regulating the bioenergetic, signaling and cell death mechanisms. Mitochondrial dysfunction in chronic metabolic and inflammatory diseases such as diabetes, kidney disease, obesity, alcoholic liver disease and cancer indicates the significance of mitochondria in maintaining normal physiology. Interestingly, these diseases exhibit extensive diversity in terms of who is susceptible and the rate of disease progression. Genetic, epigenetic and environmental factors are critical regulators of mitochondrial function. A potential relationship between mitochondrial bioenergetic function and disease severity could be exploited as a personalized predictive biomarker. At present no clinical test capable of determining the overall mitochondrial function of individuals exists. We have addressed this problem by measuring the various mitochondrial functional parameters and using these data to calculate a single mitochondrial bioenergetic health index or BHI. Although BHI incorporates interdependent but distinct parameters of mitochondrial function, the impact of physiological variables such as age, gender, ethnicity, physical activity and BMI on BHI are also not known. It is hypothesized that distinct correlations exist between the individual bionergetic parameters of BHI in healthy and diseases states.

Methods: In this study, the biological variability of BHI of human leukocytes and platelets isolated from healthy subjects and the correlation between individual bioenergetic parameters in the context of physiological variables and chronic kidney disease are demonstrated. The bioenergetic (mitochondrial respiratory) parameters of isolated leukocytes and platelets were determined using the extracellular flux analyzer (Seahorse Biosciences) and the BHI was calculated using the formula BHI = (Reserve Capacity x ATP-Linked Respiration)/(Proton Leak x Non-Mitochondrial Respiration). Multivariate statistical analysis was used to test the correlation between individual parameters. The linear correlation was determined using Pearson correlation coefficient and the significance levels were calculated.

Results: We demonstrate that BHI can stratify individuals based on their mitochondrial function (bioenergetic health). In chronic pathological conditions such as chronic kidney disease a significant decline in monocyte BHI (6.14±1.3) is observed

compared to the age-matched healthy subjects (13.08±1.13). Multivariate analysis of the bioenergetic parameters in healthy subjects show a very high correlation between basal mitochondrial respiration and ATP-linked respiration (R^2=0.92, p=<0.0001). Basal respiration is also correlated well with maximal (R^2=0.428, p<0.0001) and with non-mitochondrial respiration (R^2=0.437, p<0.0002). Although, the relationship between basal respiration and ATP-linked respiration remained unaffected, correlation between other bioenergetic parameters is breaking down in chronic kidney disease.

Conclusion: These novel findings suggest that BHI and the individual bioenergetic parameters in peripheral blood leukocytes can be used to determine the bioenergetic health of individual subjects. It is also suggested that BHI may be used to determine the disease progression in chronic kidney disease and possibly other pathological conditions. The distinct relationships between mitochondrial bioenergetic parameters suggest their potential utility in gaining insights into the mechanism of diseases with bioenergetic dysfunction. Taken together, BHI is a novel parameter that shows promise for being used as a clinical tool in developing personalized management/therapeutic strategies in patients.

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Association between functional polymorphisms in the Toll-like receptor 4 (TLR4) gene and HD severity

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Background:

Autoimmune thyroid diseases (AITDs), including Hashimoto's disease (HD) and Graves' disease (GD), are organ-specific autoimmune diseases. Disease severity can vary among patients, and it is very difficult to predict. The toll-like receptor (TLR) is an essential regulator of the innate immune system, and it functions to recognize pathogen-associated molecular patterns. TLR4 reacts with Lipopolysaccharide (LPS) as exogenous ligand and heat shock proteins and fibrinogen as endogenous ligands, and promotes cytokine production.TLR4 expression is associated with the several autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus. However, an association between TLR4 polymorphisms and the pathogenesis of AITD has not been reported. To clarify the association between TLR4 polymorphisms and AITD prognosis, we genotyped TLR4 polymorphisms.

Methods:

Among 151 HD patients who were positive for the anti-thyroid microsomal antibody (McAb) or anti-thyroglobulin antibody (TgAb), 59 developed hypothyroidism before the age of 50 and were treated with thyroxine (severe HD), 47 HD patients over the age of 50 were left untreated and demonstrated euthyroid (mild HD), and 45 HD patients could not be categorized to severe or mild HD at the time of analysis were examined. In addition, among 159 GD patients who were positive for anti-thyrotrophin receptor antibody (TRAb) at diagnosis, 68 were euthyroid, had been treated with methimazole for at least five years and were still positive for TRAb (intractable GD),42 GD patients maintained a euthyroid state and were negative for TRAb for more than two years after discontinuing the anti-thyroid drug therapy (GD in remission), and 49 could not be categorized to intractable GD or GD in remission at the time of analysis were examined. We also examined 94 healthy volunteers (control subjects) who were euthyroid and negative for thyroid autoantibodies. Written informed consent was obtained from all of the patients and control subjects. The study protocol was approved by the Ethics Committee of Osaka University. Initially, we performed direct sequencing to genotype 20 SNPs (rs144028493, rs11536888, rs1057312, rs189683599, rs1064290, rs1057313, rs1057314, rs55910231, rs56332471, rs41426344, rs1064292, rs1057316, rs55861596, rs113017335, rs7869402, rs1057317, rs41296047, rs11536889, rs60972665) from randomly selected AITD patients. Because the minor allele frequencies were >5% only for the rs41426344 and rs11536889 polymorphisms, we genotyped these polymorphisms in all AITD patients using the PCR-RFLP method.

Results:

1.In the rs41426344 polymorphism, the C allele was more frequent in the AITD and HD (5.6% and 6.4%, respectively) compared to control subjects (2.2%) (p=0.0459 and 0.0311, respectively).

2.In the rs41426344 polymorphism, the GC + CC genotypes and C allele were more frequent in the mild HD (26.7% and 14.4%, respectively) compared to the severe HD (6.8% and 3.4%, respectively) (p=0.0051 and 0.0037, respectively) and control subjects (4.4% and 2.2%, respectively) (p=0.0003 and 0.0002, respectively).

3.The distribution of the rs11536889 polymorphism was similar among all of the groups examined.

4.No association was observed between these polymorphisms and TRAb, McAb and TgAb levels or goiter size.

Conclusion:

The GC + CC genotypes of the TLR4 rs41426344 polymorphism protect against thyroid destruction and are less prone to hypothyroidism.

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Anti-PLA2R antibodies in Chinese patients with membranous nephropathy

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Objective. The prevalence of autoantibodies against phospholipase A2 receptor (PLA_2R) among Chinese patients with primary membranous nephropathy (PMN) is still not known. This study used two standardized methods to evaluate anti-PLA_2R antibody in serum to determine whether immunological reactivity reflected by antibody titer correlates with kidney function parameters.

Methods. Overall, 82 subjects with biopsy-proven primary membranous nephropathy (PMN) , 22 cases with secondary membranous nephropathy (SMN), 40 non-MN patients with established glomerulonephritis, 20 healthy volunteers were recruited from the Division of Nephrology, Nanfang Hospital, China. Anti-PLA_2R antibody in the serum of each patient was evaluated by both recombinant cell-based indirect immunofluorescence assay (RC-IFA) and enzyme linked immunosorbent assay (ELISA). Kidney function was assessed by proteinuria for 24 hours, serum albumin, blood urea nitrogen (BUN), serum creatine, serum cystatin C. We assessed the correlation between anti-PLA_2R antibody levels and clinical parameter in the PMN patients.

Results. Fifty-three patients with PMN (64.6%) were positive for anti-PLA_2R antibody. The level of antibody determined by RC-IFA ranged from 1:10 to 1:1000 and 0 to 1423 RU/ml by ELISA. The two anti-PLA_2R test systems correlated very well with each other and reached an agreement of 95.7% for PMN patients. The level of antibody detected by ELISA in patients with PMN also significantly correlated with proteinuria and nephritic-range proteinuria (> 3.5g/day).

Conclusions. Anti-PLA_2R antibody is sensitive and extremely specific for diagnosis of Chinese patients with primary membranous nephropathy. Concentration of autoantibody against PLA_2R is an ideal marker for monitoring the activity of immunological disease.

Keywords: phospholipase A2 receptor, membranous nephropathy

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The industrial food additive microbial transglutaminase is immunogenic in children with celiac disease

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Introduction: Microbial transglutaminase (mTg) is capable of cross-linking numerous molecules. It is a family member of human tissue transglutaminase (tTg), involved in Celiac disease (CD). Despite declarations of mTg safety, direct evidence for immunogenicity of the enzyme is lacking. Aims: To explore the immunogenicity of mTG compare to tTG in CD. Materials and methods: The serological activity of mTg, tTg, gliadin complexed mTg (mTg neo-epitope) and gliadin complexed tTg (tTg neo-epitope) were studied in: 95 pediatric celiac patients (CD), 99 normal children (NC) and 79 normal adults (NA). Sera were tested by ELISAs, detecting IgA, IgG or both IgA and IgG: AESKULISA® tTg (tTg), AESKULISA® tTg New Generation (tTg neo-epitope (tTg-neo)), microbial transglutaminase (mTg) and mTg neo-epitope (mTg-neo). Marsh criteria was used for the degree of intestinal injury. Results: Comparing pediatric CD patients with the 2 normal groups: mTg-neo IgA, IgG and IgA+IgG antibody activities exceed the comparable mTg ones (p<0.0001). All mTg-neo and tTg-neo levels were higher (p<0.001). tTg IgA and IgG+IgA were higher than mTg IgA and IgA+IgG (p tTg-neo IgA. Taken together, mTg-neo IgG and tTg-neo IgG correlated best with intestinal pathology (r2=0.989, r2=0.989, p<0.0001, p<0.0001, respectively). Conclusions: mTg is immunogenic in children with CD and by complexing to gliadin its immunogenicity is enhanced. Anti-neo-epitope mTg antibodies correlate with intestinal damage to the same degree as anti-tTg. Further studies are needed to explore the pathogenic potential of anti-mTg antibodies in CD.

Comparison of the reliability of celiac disease serology to reflect intestinal damage

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Introduction: In view of the increasing importance of the serological biomarkers for the screening and diagnosis of celiac disease, their differential performance, and the lack of head to head comparison, the reliability of those isolated or combined antibodies to reflect the intestinal damage in children with CD was evaluated.

Material and methods: 95 pediatric CD patients (mean age 8.3), 45 nonspecific abdominal pain children (AP) (mean age 7.3), 99 normal children (NC) (mean age 8.5) and 79 normal adults (NA) (mean age 28) were tested by the following ELISAs, detecting IgA, IgG or both, IgA and IgG: AESKULISA® Gliadin (AGA), AESKULISA® tTg (tTG; RUO), AESKULISA® DGP (DGP) and AESKULISA® tTg New Generation (Neo-epitope tTg complexed to gliadin=tTg-neo). The results were compared to the degree of intestinal injury, using revised Marsh criteria. Scatter diagrams and regression analysis comparing the 12 antibodies' optical density (OD) activities to the degree of the intestinal damage were correlated.

Results: Most of the assays were able to differentiate patients with low and high degree of intestinal damage. Comparing the different correlations between CD associated IgA and IgG antibodies' isotypes, the tTg neo IgA (r2=0.968, p<0.0025) and tTg-neo/DGP IgGs (r2=0.989, p<0.0001/ r20.985, p<0.0001, respectively) stood out, significantly, as the best indicators of the intestinal damage in CD.

The highest OD values (medium 2.94±1.2, p<0.0001) were achieved by using the tTg-neo IgA ELISA in patients with Marsh 3c.

Conclusions: It is suggested that tTg-neo IgA/IgG antibodies should be preferably used to reflect intestinal damage during screening, diagnosing and monitoring compliance in childhood CD.

Technical performance evaluation of the Elecsys® Periostin immunoassay as a companion diagnostic for the investigational asthma drug lebrikizumab

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Background: Asthma is a chronic respiratory disorder with complex clinical and biological heterogeneity and differential response to treatment. The Type 2 cytokine interleukin-13 (IL-13) plays a central role in asthma pathophysiology in a subset of patients and is being investigated as a potential therapeutic target. Periostin is a systemic biomarker of Type 2-driven asthma that can act as a surrogate biomarker for IL-13 activity in the lung and is predictive of benefit from the investigational anti-IL-13 drug lebrikizumab (Corren *et al.* NEJM 2011). The Roche Elecsys® Periostin immunoassay is being developed as a companion diagnostic for lebrikizumab in asthma. A clinical trial version of the assay was used in the Phase IIb lebrikizumab trials LUTE and VERSE to stratify patients by baseline serum periostin levels based on a cut-off of 50 ng/mL established in the previous Phase II study MILLY (Corren *et al.* NEJM 2011). Given this defined cut-off and the narrow, normal distribution of serum periostin values, strict adherence to quality control (QC) procedures and a high level of precision, accuracy, and reproducibility is required for the assay. The periostin assay has previously demonstrated robust precision during routine testing (Sherman *et al.* AACC 2014). Here, we further evaluate the technical performance of the periostin clinical trial assay and describe its QC performance over an extended period of time.

Methods: The technical performance of the periostin clinical trial assay (limits of blank [LoB], detection [LoD] and quantitation [LoQ], linearity, interferences, sample stability, and lot-to-lot comparability) was evaluated according to CLSI guidelines. The assay was further used for clinical sample testing at three external testing laboratories on four different **cobas e** 601 analyzers, using two different reagent lots, between July 2013 and November 2014. Laboratory-specific QC target values and ranges were determined based on 20 QC runs performed over 10 days. QC rules were defined for three levels of human serum samples used as QC material, and applied during clinical sample testing.

Results: Based on CLSI EP-17 procedures, using two **cobas e** 601 analyzers, the specified LoB, LoD, and LoQ was confirmed, at 2 ng/mL, 4 ng/mL, and 10 ng/mL (at 30% total allowable error), respectively. Linearity was confirmed between 4 and 160 ng/mL (CLSI guideline EP-6). No significant differences in the recovered periostin concentration were observed in the presence of a broad range of potentially interfering substances and drugs (including asthma medications), and samples were stable across a range of storage conditions and durations. The assay demonstrated good lot-to-lot comparability. The variability of valid QC measurements in the external laboratories ranged from 1.7% to 2.5% CV, and the mean relative deviation of QC levels from the global target values (bias) was between -3.2% and 2.5%.

Conclusion: The clinical trial version of the Elecsys® Periostin immunoassay demonstrated robust technical and QC performance. These results support the suitability of the assay for patient stratification in lebrikizumab clinical studies. Disclaimer: This product is not cleared or approved for use in the USA.

Adding an additional biomarker IL-18 does not improve the QuantiFERON TB test

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Background: The QuantiFERON-TB Gold In-Tube is a widely used test for mycobacterium tuberculosis (TB) exposure which measures interferon-ɤ (IFN-γ) production in peripheral blood following *in vitro* incubation with TB antigens. Although this test is widely used as a screening test, false-positive and indeterminate results are common which can lead to unnecessary follow-up testing. Interleukin 18 (IL-18), also known as IFN-γ inducing factor, is known to be a primary regulator of IFN-γ production in inflammatory reaction. The aim of this study is to evaluate if QuantiFERON test interpretation could be improved by simultaneous measurement of IL-18 and IFN-γ. **Method:** Residual materials from patient samples submitted for the QuantiFERON testing were collected. Each patient sample consisted of three conditions; a negative (Nil) control tube (containing saline), a mitogen control tube (containing PHA) and a TB antigen tube (containing a cocktail of ESAT-6, CFP-10, and TB7.7). The QuantiFERON test was performed and interpreted according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). Samples were classified into three groups according to the QuantiFERON results: TB positive (n=30), negative (n=32) and indeterminate (n=30). IL-18 concentration in each tube was determined using an electrochemiluminescence assay (Meso Scale Discovery. Rockville, MD). **Results:** A strong IL-18 release was detected in the mitogen control tube for all samples with an average of 810.8±641.4 pg/mL (Mitogen-Nil). IL-18 levels in the TB antigen tubes of all groups were significantly higher than that in the Nil control tubes (p<0.01). However, no differences in IL-18 levels were seen between different patient groups. The TB antigen induced IL-18 release (TB antigen-Nil) in the TB positive, negative and indeterminate group was 182.1±383.5, 168.7±211.8 and 214.6±413.2 pg/mL respectively. The TB antigen induced IL-18 release in the TB infected patients was similar to that in the non-infected subjects (p>0.05). Additionally, no correlation was found between the TB antigen-stimulated IFN-γ and IL-18 release in the TB-positive patients. To normalize possible impact from variation of cell number and immune response, we further calculated the ratio of TB antigen-induced to mitogen-induced IL-18 release. This ratio remained similar between the TB positive and negative patients. ROC analysis showed that the AUC was 0.48 (TB antigen induced IL18 release) and 0.49 (Ratio: TB antigen/Mitogen induced IL18 release) in differentiation of the TB positive and TB negative patients. **Conclusion:** No significant increase of IL18 secretion was elicited by TB antigen in TB infected patients. Quantitation of IL18 in addition to IFN-γ is not useful in improving QuantiFERON test interpretation.

Prevalence and distribution of IgG, IgA and IgM Gammopathies in samples obtained from a large reference Laboratory in Brazil.

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Background: Monoclonal gammopathies (MG) are a group of disorders characterized by monoclonal proliferation in plasma cells resulting in the production of monoclonal immunoglobulin or immunoglobulin fragment (M protein), which can be detected in serum in the form of a band or monoclonal component (M component). Immunoglobulins or antibodies typically consist of two heavy polypeptide chains and two light polypeptide chains. There are five types of heavy chains named IgG, IgA,

IgM, IgD, and IgE; and two light chain different types, called kappa and lambda. Thus, MG can be classified by the type of light and heavy chains produced. This identification is possible due to specific techniques that are able to define the type of abnormal proteins, identifying the heavy and light chains involved, such as immunofixation, which combines two other techniques: immunoprecipitation and electrophoresis. Confirmation of the presence of monoclonal protein is essential for differentiation of monoclonal gammopathies and polyclonal gammopathies. The first is a neoplastic or potentially neoplastic proliferation, and the last, results from infective or inflammatory processes.

Objective: We aimed to access the prevalence and distribution of IgG, IgA and IgM gammopathy in a large reference Laboratory in Brazil.

Methods: We identified from May 2013 to June 2014, 1548 serum immunofixation assay results from a large brazilian reference laboratory database. The types of chains were detected with 9IF immunofixation methodology, in conjunction with the semi-automatic Hydrasys system, from Sebia® manufacturer.

Results: Monoclonal gammopathy was detected in 452 samples in a total of 1548 samples analyzed, the prevalence was 29.20% in the present study, the highest prevalence of monoclonal component were observed in males, aged from 61 to 70 years old (40.34%). In gammopathy distribution, the most prevalent isotype found was IgG kappa (43.14%), followed by IgG lambda (26.33%), IgA kappa (14.60%), IgA lambda (12.17%), IgM, kappa (2.88%) and the less common IgM lambda (0.88%).

Conclusion: Among patients referred by physicians to our clinical laboratory with a prescription of serum-immunofixation, gammopathy was found in 29.20% of this population. IgG class is the most prevalent, followed by IgA and IgM and this results are being consistent whit the literature. The analysis option used for the diagnosis of MG is a technique which has high sensitivity and have wide application in the identification of M protein, normally present in small amounts, which are difficult to detect by other methods. A correct identification of monoclonal component class is important because each type is associated with little differences in disease patterns, being essential for guidance and monitoring of therapeutic or treatment efficacy.

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A rare case of paraneoplastic limbic encephalitis associated with squamous cell lung carcinoma

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Background: Limbic encephalitis (LE) is a paraneoplastic syndrome that is often associated with small cell lung cancer (SCLC), breast cancer, testicular tumors and thymoma. The common clinical manifestations of LE are subacute onset, cognitive dysfunction, seizures and psychiatric symptoms. The main intracellular antigens related to limbic encephalitis are Hu, Ma2, and less frequently CV2/CRMP5 and amphiphysin.

Methods: A 79 years old man with behavioral disorders, seizures and fever was admitted to the Emergency Service of our Hospital. The fever was confirmed (37.2 ºC), CT scan showed residual ischemic brain injury. The diagnosis was subacute encephalitis. However, the patient's condition deteriorated with aggressiveness, agitation and three episodes of oculocephalic version. The patient was admitted in the Service of Neurology to study the origin (infectious, autoimmune or neoplastic) of the episodes of subacute encephalitis.

Results:

1. Biochemistry study

LDH (696 U/L) and PCR (18.4 mg/dL) altered without significant alterations of the others parameters of the biochemistry. Normal thyroid function.

2. Hematology study

CBC without significant alterations.

3. Tumor markers

NSE (Neuron-Specific Enolase)=27.3 ng/mL.

AFP, CEA, CA-125, CA-15.3, CA-19.9, B-HCG and PSA in normal ranges.

4. Microbiological analysis (Study of meningitis and viral encephalitis)

PCR negative for Herpes simplex, Varicella-zoster, Toscana virus, Enterovirus.

5. Autoimmune study

Antinuclear antibodies (ANA), DNA and ENAs screening: negatives

Onconeuronal antibodies: Positive anti-amphiphysin antibodies 1/100 and confirmed by immunoblot assay. The presence of this antibody is associated to paraneoplastic LE. The presence of this antibody allow us to diagnose the patient's symptoms as LE.

6. Chest X-rays

Normal, without presence infiltrates or masses.

7. Thoraco abdominal CT SCAN

Condensation at left lung and presence of adenopathies in mediastinum and abdomen suggesting lung carcinoma.

8. Biopsy sample by bronchoscopy

A biopsy sample of bronchial fragments of the mucosa showed the presence of tumoral cells CK5/6+, P63+, TTF1- and CK7- associated to scamous cell lung carcinoma.

9. Diagnosis of the patient

With previous findings, the diagnosis of the patient was paraneoplasic limbic encephalitis with anti-amphiphysin antibodies positives associated to scamous cell lung carcinoma.

Conclusion: The presence of anti-amphiphysin antibodies allows us to diagnose a scamous cell lung carcinoma. The paraneoplastic antibodies appear to be a useful tool for diagnosing a neurological disorder as paraneoplastic and for indicating the probable type of underlying tumor.

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The Grace Bio-Labs ArrayCAM Multiplex Protein Microarray System surpasses the diagnostic throughput and accuracy shortfalls of ELISA and Multiplex Bead Assays

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Background: Simultaneous multiplexed profiling of protein markers will become standard method for physicians to more accurately diagnosis disorders and/or monitor therapeutic responses, yielding improved survivability or manageability of disease. Autoimmune connective tissue diseases are an example, where profiling multiple auto-antibodies to dsDNA and numerous extractable nuclear antigens (ENA's) defines the presence of specific disease subset(s). Here ELISA is customarily the standard screening method, but lacks the through-put economy and diagnostic power of multiplex applications. This has driven market appraisal of Luminex, providing through-put economy and multiplexing diagnostic advantages, but exhibiting deficiencies in accuracy and consistency.

Grace Bio-Labs has developed the ArrayCAM Microarray System for multiplexed protein profiling consisting of complimentary optimized components, providing the solution to these predicate device shortfalls. Here, we illustrate efficacy of the ArrayCAM Microarray System for high through-put, sensitive and specific multiplexed detection of protein biomarkers, providing next-generation diagnostics.

Method: The multiplex Grace BioLabs Microarray Assay System components begin with antigens arrayed onto ONCYTE high binding porous nitrocellulose slides producing multiple co-localized assay reaction sites within each well. Sample analyte detection is achieved using single wavelength excitation quantum nanocrystals, providing extremely high signal emission in discrete wavelength bands. This enables multi-color multiplexing readable with an economical single laser image acquisition instrument. This data capture and analysis is achieved using the ArrayCAM imager and automated software, which rapidly acquires and analyzes data, then provides objective test reports, thereby eliminating the subjective influence of technical operators. In this illustration we simultaneously measure presence of serum autoantibodies to SM, SMRNP, SSA60 kDa, SSA52 kDa, SSB, SCL-70, JO1, RIBO-P and dsDNA (semi-quantitatively) using multiplex reverse capture immunoassay methods. Test wells also contain human-IgG calibrators, anti-human IgG controls, interference controls and alignment fiducials. To demonstrate performance, 64 human ANA disease positive samples were sourced from commercial vendors characterized for anti-dsDNA and anti-ENA autoantibodies via ELISA (Inova, ImmunoConcepts). Samples were then screened with the ArrayCAM Microarray System and data expressed as semi-quantitative binary auto-antibody positivity established by known positive/negative cut-offs determined from predicate device sample characterizations. Anti-dsDNA measurements were reported semi-quantitatively using regression of sample signal against Hu-IgG intra-well curve calibrators at known concentrations.

Results: The microarray performed to clinical standards with observed specificity ranging through 90.6% to 100% (mean, 94.9%) and sensitivity ranging through 66.7% to 100% (mean, 93.3%). Sample measurements for anti-dsDNA exhibit semi-quantitative measurements correlating to those produced by ELISA. Also, the ability of the microarray to integrate unique controls and multiple antigen epitopes illustrates potential false negative and positive measurements by ELISA.

Conclusions: The Grace Bio-Labs ArrayCAM Protein Microarray System provides the economical and diagnostic advantages of multiplexing not possible with single-plex ELISA along with superior sensitivity and specificity than Luminex by virtue of

unique assay controls. The shortfalls of these two predicate systems are overcome while delivering clinical performance specifications with familiar, easy-to-use methods, requiring no significant technical training.

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Evaluation of Anti-HTLV (I/II) CMIA testing in a comparative study with results of HTLV blot assay: Anti-HTLV (I/II) CMIA gray zone needed?

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Background: Human T-lymphotropic virus (HTLV)-1 and HTLV-2 were the first oncogenic human retroviruses identified in the early 1980's. HTLV-1/2 are retroviruses linked etiologically to various human diseases, and both of them can be transmitted by vertical route, sexual intercourse, blood transfusion and intravenous drug use. This infection has a worldwide distribution, with an estimate of up to 15-20 million people affected.

Diagnosis and screening is important in protecting the safety of blood products and in the disease diagnostic, but better specificity and sensitivity assays are still necessary. This study aims to suggest the creation of a gray-zone to the CMIA HTLV based in a comparison of a gold standard HTLV Blot test.

Methods: Using the database of a clinical laboratory, we assessed 1103 patients results of HTLV during the year of 2014 from Brazil. The tests performed were HTLV Blot (MPD HTLV Blot 2.4) and CMIA (Architect ® rHTLV-I/II, Abbott), following strictly manufacturers' instructions and evaluated with internal control. For CMIA testing, firstly results were interpreted according to package insert (considering cut-off at 1.0 S/CO), and secondly, gray-zone intervals were created and evaluated to determinate inconclusive results.

Results: From 1103 samples tested by HTLV Blot, 799 showed negative, 243 positive and 55 were inconclusive. When samples were tested by CMIA we obtained 757 negative and 346 positive results.

Afterwards CMIA results were reclassified allocating the results in seven different groups as shown on the table 1.

		HTLV Blot		
	Results (S/CO)	POS	INC	NEG
rHTLV I/II CMIA	0,0 - 1,0	0	16	741
	1,0 - 5,0	0	13	56
	5,0 - 10,0	0	10	0
	10,0 - 20,0	0	3	2
	20,0 - 50,0	6	11	0
	50,0 - 100,0	80	2	0
	> 100,0	163	0	0

Table - Classification groups

Conclusion: CMIA and HTLV blot results showed that we had a significant number of discrepant results. We think that the reclassification is necessary, thus we suggested a new distribution for the CMIA results creating a gray zone of 1,0 - 20,0 S/CO for allocating inconclusive results. This new gray zone makes possible a reliable and assertive diagnostic, reducing substantially the possibility of false negative result.

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Reduction of Immunoglobulin Carry-over on an Automated Tecan Pipetting Platform

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Background: Measurement of IgG responses to the *Streptococcus pneumoniae* vaccine are often assessed for patients with suspected immunodeficiency. The degree of response to a vaccination can vary significantly. Because patients with very high concentrations of antibody may be analyzed together with patients having undetectable responses, carry-over on automated pipetting platforms is a concern. When carry-over is identified, the affected patient sample must be repeated, adding cost and time to the testing process. The purpose of this study was to identify a procedure that would eliminate carry-over in a 23-plex *Streptococcus pneumoniae* antibody assay. Methodology: *Streptococcus pneumoniae* antibodies are measured by dispensing 5 serial dilutions of each patient sample into a 96-well vacuum filter plate via a Tecan

Freedom Evo. A mixture of Streptococcal polysaccharide-conjugated microspheres (representing each of the 23 polysaccharides in the Pneumovax-23 vaccine) is added to each patient dilution. Following incubation and washing, a fluorescein-conjugated anti-hIgG is added to each dilution. After a second incubation and wash, the mean fluorescence intensity (MFI) for each bead is measured on a Luminex platform. Calculation of patient results is performed using the statistical analysis program StatLia (Brendan Scientific). A recent publication demonstrated that 0.17M NaOCl washes could eliminate carry-over of IgG molecules on the Tecan Freedom Evo platform (JALA 10.2010,p379-389). This modification was applied to our assay, with a 0.17M NaOCl wash of the probes between samples and immediately prior to the water rinse step. To assess the impact of carry-over, aliquots of relatively low and high concentrations were placed strategically throughout a run to induce carry-over. Observed carry-over, carry-over limit, and carry-over ratio were calculated for each serotype. Observed carry-over was calculated as the mean differences of the low concentrations followed by high samples and the low concentrations followed by low samples. The carry-over limit equals 3 times the standard deviation of the mean difference of the low concentration when followed by a low sample. The carry-over ratio equals the observed carry-over/carry-over limit. A method comparison was conducted by assessing the *Streptococcus pneumoniae* antibody concentrations in patient samples (n=54) performed with and without the 0.17M NaOCl wash. The results for the 23 serotypes were compared by linear regression. Patient sample repeat rates were calculated pre and post NaOCl wash (n=2441). Results: The average carry-over ratio for all 23 serotypes before the addition of the NaOCl wash was 3.5. After the addition of NaOCl, the ratio was reduced to 0.2, indicating that the majority of the carry-over had been eliminated. . Linear regression analysis showed slopes ranging from 0.857 to 1.067 for the 23 serotypes and correlation coefficients ranging from 0.952 to 0.998, demonstrating that the NaOCl wash did not affect the analytical performance of the assay. The elimination of carry-over has reduced our repeat rate from 8.3% to 6.0% which equates to about 600 fewer repeats per year. Conclusion: Carry-over can be a source of laboratory errors and identifying it is sometimes difficult. Implementation of a NaOCl wash may be effective in mitigating carry-over of immunoglobulins on automated pipetting platforms.

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Evaluation of the Analytical Performance of Multiple Optilite Systems under routine conditions

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Background: The Binding Site Optilite® system is a fully integrated immunoturbidimetric analyser that has been specifically optimised to undertake specialised protein assays. The intention of this study was to evaluate the performance characteristics of the analyser under simulated routine conditions using multiple analysers, assays and operators over a prolonged period of time. **Methods:** Four Optilite analysers were used by a total of 4 operators with 12 analytes - IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgM, C3c, C4 Beta-2-Microglobulin and the Freelite® Kappa and Lambda. The testing occurred over a 2 week period utilising a workload derived from a high volume laboratory (280 - 350 tests/day, 80-110 samples/day). All systems and reagents were final and operators were trained according to the manufacturer's final procedures. Testing was carried out using the Quality control materials provided for the precision testing and patient samples for the comparability testing. Samples were a mixture of both native and contrived materials in order to span the measuring ranges and challenge dilution capabilities of the system. **Results:** For each analyte, precision was calculated over the 10 day period and the composite standard deviation and CV derived across all 4 systems. Similarly for each analyte, patient samples were correlated using Passing-Bablok regression - all system combinations were examined. Agreement was estimated using Bland-Altman. **Conclusion:** All assays on all the Optilite Systems demonstrated excellent precision. Similarly correlation between systems was very good and showed homogeneity of performance. Under these demanding conditions, the Optilite systems proved to be robust and reliable

Analyte	QC material results			Sample results	
	QC	Mean	CV (%)	Sample range	Range of r*
Freelite Kappa	High	26.418 mg/L	4.0	0.991 – 1189mg/L	0.767 – 0.995
	Low	14.924 mg/L	5.1		
Freelite Lambda	High	61.934 mg/L	7.0	1.37 – 14.248mg/L	0.761 – 0.983
	Low	29.132 mg/L	5.4		
IgG1	High	7122.160 g/L	4.3	1628 – 5392.6g/L	0.925 – 0.965
	Low	2938.048 g/L	6.9		
IgG2	High	4169.356 mg/L	3.2	1093.39 – 4480.43mg/L	0.983 – 0.997
	Low	2394.085 mg/L	3.9		
IgG3	Elevated	1642.858 mg/L	5.1	225.97 – 1145.28mg/L	0.926 – 0.978
	High	701.955 mg/L	5.5		
	Low	350.000 mg/L	6.7		
IgG4	Elevated	1868.307 mg/L	3.5	92.71 – 2527.53mg/L	0.999 – 1.000
	High	433.890 mg/L	3.3		
	Low	237.559 mg/L	3.2		

Range of r is calculated from 6 combinations of platform comparisons by Pearson Product-moment correlation coefficient.

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Total Vs Antigen Specific Pneumococcal Antibody Response: A Comparison of Two Different Assay Types

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Background: The absence or modulation of antibody response to Pneumococcal pneumoniae (PCP) antigens may be observed in patients with an immunodeficiency. Different types of assays have been developed to evaluate Pneumococcal antibody responses. Commercial assays generally measure total PCP antibody titer, whereas some laboratory developed tests (LDT) measure antibody responses to specific PCP serotypes.

Objective: To correlate results of a commercially available assay that renders a total PCP antibody result to an LDT assay that differentiates individual antibody responses to specific Pneumococcal antigens.

Methods: 151 serum specimens were interrogated using the Anti-PCP IgG EIA (The Binding Site, UK) and results were compared with historic data obtained using a Luminex™-based LDT (Mayo Clinic) which quantifies individual responses to 23 common Pneumococcal antigens.

Results: There was moderate agreement between the summated serotype results of the LDT (Median 108.4, min 4.5 - 1764.7 max, average 232.7, μg/ml) and the total anti-PCP EIA (Median 50.7, min 5.8 - max 1055.0, average 146.8, μg/ml); (n=151 Pearson's Correlation 0.61, linear regression R2 0.37).

In a second analysis, Mayo-derived, sero-specific cut-offs were applied to each of the 23 individual Pneumococcal antibody results and the percentage of Pneumococcal antibodies rendering a positive result was determined for each of the 151 serum specimens. When these interpretations were applied to the LDT results and compared to the total anti-PCP EIA results, Pearson's correlation showed a relationship of 0.58.

In an additional comparison, Mayo-derived, sero-specific cut-offs were applied to the results of the LDT assay. Specimens rendering positive results in ≥50% or <50% of the Pneumococcal antigens tested were considered PCP responders and PCP non-responders respectively. For the total anti-PCP EIA results, specimens rendering a result ≥100 μg/ml or <100 μg /ml were considered PCP responders and PCP non-responders respectively. The 100 μg/ml cut-off was empirically determined and not optimized in context of clinical outcome. When these definitions were applied to the 151 specimens interrogated, 39 and 73 specimens rendered results consistent with the responder or non-responder definitions on both assays respectively. Collectively these data demonstrate a concordance of 74.2% among the LDT assay and the total anti-PCP EIA. Thirty-nine specimens demonstrated discordant results with 22 and 17 specimens rendering (non-responder total anti-PCP EIA/ responder LDT assay) and (responder total anti-PCP EIA/non-responder LDT assay) respectively.

Conclusion: The analytical correlation of results obtained using the Anti-PCP IgG EIA (The Binding Site, UK), which renders a total Pneumococcal antibody result, and a Luminex-based LDT (Mayo Clinic), which differentiates individual antibody responses to specific Pneumococcal antigens, display varying agreement, depending upon the interpretation criteria applied to the results. The establishment of clinically optimized cut-offs for the total anti-PCP EIA may improve the concordance of the assays.