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-HGG 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 SPL 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=c*x^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.

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


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 1,000mcg/mL were evaluated over multiple runs to establish LOD, LOQ, and analytical measurement 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 optica patients receiving eculizumab previously quantified 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 30 minutes; 55°C. A 200mcL 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 peak was manually integrated. 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%); 65mcg/mL (7%); 85mcg/mL (12%); and 300mcg/mL (13%). 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 other mAbs into serum. All were chromatographically separated and identified by their unique molecular masses.

Analytical Validation of an ELISA for the Quantitation of Calprotectin 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 secreted 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 HT 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 PhCal™ ELISA kit (Eurospiral SpA). The reference range (<120 µg/g) was assessed using extracts from healthy donors (n=20). Linearity was assayed by diluting 4 extracts with high calprotectin concentrations (259.5 to 1,211.2 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 LOD was 0.130 optical density (O.D.). The LOQ 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. Further testing may be useful prior to more invasive procedures (e.g. colonoscopy, endoscopy) used to evaluate patients suspected of IBD.
In our cohort, high CSF-FKC values above 0.38 mg/L increased the risk of conversion. Conclusion: Furthermore, high FLC levels are also strong predictors of positive OCGB criteria. MRI and OCGB index. Further results for both groups are presented in table 1.

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 multiple sclerosis (MS). Methods: 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: Cerebrospinal Fluid Free Kappa Chains as a Possible Biomarker to Assay Risk Conversion to Multiple Sclerosis

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Background: Immunology

Using a cut-off value of 0.38 mg/L determined from the mean CSF-FKC levels of patients to MS, and compare with other parameters: oligoclonal IgG bands (OCGB), IgG index and magnetic resonance imaging (MRI) criteria used in clinical practice.

Results: Using a cut-off value of 0.38 mg/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<0.001). 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.
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, 5.6, 5.3, 3.1 and 0.87%, 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.

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 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 chemotherapy and radiation. He has anaemia 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 Oligonucleotide Banding Electrophoresis in CSF: Method Comparison with Quantitative Free Light Chains and Accurate Molecular Mass Measurement of Immunoglobulins


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⁻, N= 19) by IgG-IEF (Helena SPIIF 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 (mIARM)). 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⁻ 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=0.055). MicroLC-ESI-Q-TOF oligoclonal profiles were in 100% agreement with IEF. In the OCB⁺ 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 intratherically.

Cryoprecipitate as a Quality Control Material for Cryoglobulin Analysis


Background: Cryoglobulins are serum immunoglobulins that precipitate at a temperature less than 37°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. We searched 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 redissolved 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.
Background: Systemic Lupus Erythematosus is associated with a large spectrum of autoimmune disorders, but currently there is no reliable serologic diagnosis. Each autoimmune 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™ 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 ImmunoArray. 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 ImmunoArray 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™ chip.

Results: The SLE-Key TM classifier 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™ 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-KeyTM in direct comparison to standard serologic testing in SLE patients is ongoing.

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

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 51Cr 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, Sangnam, Korea), which detect NK cell activity by measuring the secretion of IFNγ from 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 (R² = 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 correlate 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 compatible than traditional NK cell activity measurement, could be a feasible method to estimate and monitor the NK cell function in clinical setting.

Profilling 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 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 and inside the cells. Sialidase activity was measured to confirm the change in SA amounts in the culture medium.

Results: After LPS 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 (1.590.5)×103 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...
Immunology

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

A-222

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


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, IgG 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 anti-human-BCMA detection Ab was added. Human serum IgA and IgG levels were determined in MM patients using nephelometry (Immage 800, Beckman Coulter). Hyveit® Assays (Binding Site) were used to quantify the levels of heavy-light chain isofrom 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/6 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-G-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 (≥ 100 ng/ml) correlated with below normal levels of uninvolved IgG in IgA MM and uninvolved IgG in IgG MM, whereas lower BCMA levels (< 100 ng/ml) correlated with normal uninvolved levels (P = 0.0001). Using the Hyveit® 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.

A-223

Comparison between single 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 measure 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, Wallfording, 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 4 cytokines total. The singlex ELISA accommodated 80 samples per plate, excluding blanks, calibrators and quality controls (QC’s), 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: R² = 0.98 for IL-1β, 0.98 for IL-6. Assays with different antibody sources showed poor correlation: R² = 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.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Inter-assay CV (number of QC’s)</th>
<th>AMR (pg/mL)</th>
<th>Number of samples outside AMR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>ELISA</td>
<td>Cyplex</td>
<td>ELISA</td>
</tr>
<tr>
<td>4.1 - 8.4%* (20)</td>
<td>8.0% (6)</td>
<td>0.125 - 3</td>
<td>0.21 - 2000</td>
</tr>
<tr>
<td>IL-10</td>
<td>9% (30)</td>
<td>6.2% (6)</td>
<td>0.156 - 10</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6.6% (23)</td>
<td>6.2% (6)</td>
<td>0.50 - 30</td>
</tr>
</tbody>
</table>

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

A-224

Analytical Evaluation of The Helena SPIFE 3000 Electrophoresis/ Immunofixation System


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 gels 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 (r² = 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 lamabdas in urine using SPIFE.
Conclusions: For routine protein electrophoresis, the results of our evaluation showed that Helena SPIF 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 SPIF 3000 is prone to miss some monoclonal free lamids. 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 SPIF 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.

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 | Apoptosis | Benadryl |
|                 | Yes (n=20) | No (n=142) | Yes (n=21) | No (n=157) | Yes (n=67) | No (n=82) |
| Tryptase, ng/mL | Mean 63 | 60 | 84 | 13 | 20 | 14 | 15 |
| p-value | <0.0001 | | | | | |
| NMH, ng/mg cre | Mean 54 | 54 | 72 | 103 | 207 | 231 | 208 |
| p-value | <0.0001 | | | | | |
| 2,3-BPG, pg/mg cre | Mean 1517 | 500 | 84 | 955 | 977 | 852 | 1076 |
| p-value | <0.0001 | | | | | |
| LTE4, pg/mg cre | Mean 205 | 105 | 42 | 101 | 179 | 152 | 164 |
| p-value | <0.0001 | | | | | |

B-225
Application of Bioenergetic health index (BHI) in Healthy individuals and Chronic Kidney Disease

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 this study of BAL is helpful to discriminate between interstitial lung diseases
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 progression, 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, rs19683599, rs1064290, rs1057313, rs1057314, rs55910231, rs56332471, rs41426344, rs1064292, rs1057316, rs55861596, rs13017335, rs7869402, rs1057317, rs41296047, rs11356889, rs6097265) 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.

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=0.003 and 0.0002, respectively).

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.
Tuesday, July 28, 9:30 am – 5:00 pm

Immunology

A-232

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® Gliladin (AGA), AESKULISA® Tg (TG; RUO), AESKULISA® DGP (DGP) and AESKULISA® Tg New Generation (Neo-epitope Tg complexed to gliadin= Tg-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 Tg(neo) IgA (r=0.968, p<0.0025) and Tg(neo)/DGP IgGs (r=0.989, p=0.0001 / r=0.985, 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 Tg(neo)-IgELISA in patients with Marsh 3C.

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

A-233

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 Ib 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 colabs e 601 analyzers, using two different reagent lots, between July 2013 and November 2014. Laboratory-specific QC target values and stability, and lot-to-lot comparability) was evaluated according to CLSI guidelines. 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.

A-234

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-γ (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 (QUIAGEN 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 810.6±641.1 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.

Conclusions: No significant increase of IL-18 secretion was elicited by TB antigen in TB infected patients. Quantification of IL-18 in addition to IFN-γ is not useful in improving QuantiFERON test interpretation.

A-235

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 light polypeptide chains. There are five types of heavy chains named IgG, IgA, IgM, IgD and IgE.

Results: Based on CLSI EP-17 procedures, using two colabs e 601 analyzers, the specified LoB, LoD, and LoQ was confirmed, at 2 mg/mL, 4 mg/mL, and 10 mg/mL (at 30% total allowable error), respectively. Linearity was confirmed between 4 and 160 mg/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.
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 °C). 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 oculecephalic 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: 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.

Hematology study
CBC without significant alterations.

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.

Microbiological analysis (Study of meningitis and viral encephalitis)
PCR negative for Herpes simplex, Varicella-zoster, Toscana virus, Enterovirus.

Autoimmune study
Antinuclear antibodies (ANA), DNA and ENAs screening: negatives
Onconeural 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.

Immunology
IgM, IgG, 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 with 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.

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


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 Lumixin, 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 analyze 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 image 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, J10, RIBO-P and dsDNA (semi-quantitatively) using multiplex reverse capture immunooassay 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, Immunocorcepts). 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 singleplex ELISA along with superior sensitivity and specificity than Lumines 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.

**A-238**

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 (MDP 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 cutoff at 1.0 S/CO), and secondly, gray-zone intervals were created and evaluated to determine 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.

<table>
<thead>
<tr>
<th>HTLV Blot</th>
<th>Results (S/CO)</th>
<th>POS</th>
<th>INC</th>
<th>NEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHTLV I/II CMIA</td>
<td>0.0 - 1.0</td>
<td>0</td>
<td>16</td>
<td>741</td>
</tr>
<tr>
<td></td>
<td>1.0 - 5.0</td>
<td>0</td>
<td>13</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>5.0 - 10.0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10.0 - 20.0</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20.0 - 50.0</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50.0 - 100.0</td>
<td>80</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>&gt; 100.0</td>
<td>163</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Table - Classification groups**

**Conclusion:** CMA 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.

**A-240**

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

S. Thouless, J. Overton, P. Kenny, S. Harding. The Binding Site Group Ltd, Birmingham, United Kingdom

**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 analytic, 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.

**A-239**

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

L. J. Ouverson, M. R. Snyder. Mayo Clinic, Rochester, MN

**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-IgG 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 Stat.Ja (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=2 441). 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.
Immunology

<table>
<thead>
<tr>
<th>Analyte</th>
<th>QC</th>
<th>Mean</th>
<th>CV (%)</th>
<th>Sample range</th>
<th>Range of ( r^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freelite Kappa</td>
<td>High</td>
<td>26.418 mg/L</td>
<td>4.0</td>
<td>0.991 – 1189 mg/L</td>
<td>0.767 – 0.995</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>14.924 mg/L</td>
<td>5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freelite Lambda</td>
<td>High</td>
<td>61.934 mg/L</td>
<td>7.0</td>
<td>1.37 – 14.248 mg/L</td>
<td>0.761 – 0.983</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>28.132 mg/L</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>High</td>
<td>7122.160 g/L</td>
<td>4.3</td>
<td>1628 – 5392.6 g/L</td>
<td>0.925 – 0.965</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>2938.048 g/L</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2</td>
<td>High</td>
<td>4169.356 mg/L</td>
<td>3.2</td>
<td>1093.39 – 4480.43 mg/L</td>
<td>0.983 – 0.997</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>2394.085 mg/L</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG3</td>
<td>Elevated</td>
<td>1642.858 mg/L</td>
<td>5.1</td>
<td>225.97 – 1145.28 mg/L</td>
<td>0.926 – 0.978</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>701.955 mg/L</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>350.000 mg/L</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG4</td>
<td>Elevated</td>
<td>1868.307 mg/L</td>
<td>3.5</td>
<td>92.71 – 2527.53 mg/L</td>
<td>0.999 – 1.000</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>435.890 mg/L</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>237.559 mg/L</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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.