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# Voordrachten

Samenvattingen van de voordrachten tijdens het 59e Congres van de Nederlandse Vereniging voor Klinische Chemie en Laboratoriumgeneeskunde, op 12 en 13 april 2006 te Lunteren

## Sessie 1 Analytisch

9.00 - 9.15 uur

### Lamotrigine in dried bloodspots by HPLC analysis

J.W.P.H. SOONS, M.L. van BREE, J.H. COUMOU, J.A.R.J. HULSMAN
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*Introduction:* The anti epileptic drug, lamotrigine, is standard monitored in plasma by HPLC. We designed an assay inwhich lamotrigine is measured in dried bloodspots and compared this assay with the standard plasma analysis.
*Methods:* Lamotrigine is measured in plasma or dried bloodspots obtained from patients blood by HPLC. Discs of 1/4 inch were purched of paper (Schleicher and Schuell). Samples (10 µL) of blood, lamotrigine and/or internal standard were added to the disc. The sample size of the plasma analysis is 200 µL. Lamotrigine and the internal standard were extracted out of the plasma sample or the dried bloodspot by 4 mL dichloromethane(97%) / isopropanol(3%). After evaporation and reconstitution of the residue in 200 µL mobile phase solution 10 µL is injected to the HPLC system.
*Results:* The concentration of lamotrigine in 17 patients is measured in the following different bloodcomponents: whole blood, lysed whole blood, red blood cells, lysed red blood cells and plasma (reference). No significant difference in the lamotrigine concentration is found in these bloodcomponents when compared to the plasma concentration. The between run VC of the lamotrigine analysis using the dried bloodspots is measured in three patients. The results are 10.7%, 8.9% and 10.6% at a lamotrigine concentration of respectively 3.8 mg/L, 6.5 mg/L and 9.3 mg/l. Comparison of the lamotrigine concentration in dried bloodspots and in plasma is obtained by measuring the concentration in 13 patients recieving lamotrigine. Passing and Bablock analysis of these data shows no significant difference between the two assays.
*Conclusion:* Lamotrigine can be measured in dried bloodspots by HPLC analysis. The lamotrigine concentration measured in the dried bloodspots correlates well (y=1.05x + 0.06, r=0.994) with the concentration measured in plasma.

9.15 - 9.30 uur

### Novel urine hepcidin assay by mass spectrometry

E.H.J.M. KEMNA[1], H. TJALSMA[1], C. LAARAKKERS[1], E. NEMETH[2], J.L. WILLEMS[1], D.W. SWINKELS[1]
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*Inleiding:* Hepatic peptide hormone hepcidin is the central regulator of iron metabolism and mediator of anemia of inflammation. To date, only one specific immuno-dot assay to measure hepcidin in urine had been documented (1).
*Methode:* Here we report an alternative approach for quantification of hepcidin in urine by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).
*Resultaat:* Peptide peaks were detected corresponding to the three forms of hepcidin normally found in urine. The identity of the peptide peak equivalent to hepcidin-25 was confirmed using synthetic human hepcidin-25.Validation of our MS data on samples with various hepcidin levels showed a strong correlation with previous immuno-dot assay results (Spearman R = 0.9275, P<0.0001). Most importantly, this hepcidin assay clearly discriminates between relevant clinical iron disorders.
*Conclusie:* In conclusion, this novel MS urine hepcidin assay is easy to perform and available to a wide audience. This enables the implementation of hepcidin measurements in large clinical studies.

*Literatuur:* Nemeth et al. Blood 2003; 101: 2461-2463.

9.30 - 9.45 uur

**Standardization of calibration and quality control using Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometry**

J.A.P. BONS, D. de BOER, M.P. van DIEIJEN-VISSER, K.W.H. WODZIG
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*Introduction:* Protein profiling by Surface Enhanced Laser Desorption Ionization-Time of Flight-Mass Spectrometry (SELDI-TOF-MS) is gaining importance as a diagnostic tool for a whole range of diseases. This report describes a quality control (QC) procedure, which acts prospectively by checking the calibration before starting profiling experiments
*Methods:* A well-defined protocol for calibration of the Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS) part of the Protein Biosystem IIc (PBSIIc) instrument was established, using commercial QC samples containing independent certified standards and by determination of acceptance criteria. The QC samples consisted of the Proteomass MALDI-TOF-MS standards insulin, apomyoglobin, and albumin with [M + H]+ ions at m/z 5734.51, 16,952.27 and m/z 66,429.09 Da, respectively. Instrument calibration was performed externally every week with the standards provided by the manufacturer.
*Results:* According to the acceptance criteria defined in this study, data points should be in the established range of the process mean ± 2 standard deviations for the mass-to-charge ratios (m/z values), peak intensities, signal-to-noise ratios (S/N), and peak resolutions for insulin, apomyoglobin and albumin in the QC samples. Moreover, it was demonstrated that the pipetting variability in the handling of the QC sample significantly contributed to systematic errors and that spotting of a larger volume of QC sample resulted in a better reproducibility. Other potential sources of variation included chip variability, crystallization of the energy absorbing matrix, and laser detector variability over time.
*Conclusion:* Stringent quality control of the calibration part of the SELDI-TOF-MS experiments prevents unreliable data acquisition from the very start, because when the data of the QC sample exceeds the acceptance criteria, actions needs to be undertaken before starting new protein profiling experiments.

9.45 - 10.00 uur

**A reliable, sensitive and semi quantitative real time PCR assay to detect the JAK2 V617F mutation in blood from patients with myeloproliferative disease.**

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*Multidisciplinary Laboratory for Molecular Diagnostics[1], Department of Hematology[2], Laboratory for Clinical Chemistry and Hematology[3], Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands*

*Introduction:* Diagnosis of the myeloproliferative disorders, polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF) is difficult due to lack of diagnostic markers. Recently the acquisition of a mutation in the Janus kinase 2 (JAK2) gene by hemopoietic cells has been described as a genetic defect underlying myeloproliferative disorders (Baxter EJ et al 2005, James C et al 2005, Kralovics et al 2005). The mutation leads to constitutive activation of JAK2, a tyrosine kinase involved in cytokine receptor signalling.
*Methods:* Because of the clinical importance of this mutation (JAK2V617F) in diagnosing myeloproliferative disorders and its relevance for disease progression, we developed two assays to detect JAK2V617F.
*Results:* Both tests -RFLP and real time PCR- appeared more sensitive than direct sequencing, the method currently used to detect JAK2V617F. The RFLP assay, although certainly suitable to detect JAK2V617F, remained troublesome due to poor performance of the restriction enzyme. The real time PCR assay, in contrast, provided a quick (within half a day), robust and simple method to detect JAK2V617F in quantities below 1% amongst wild type DNA.
*Conclusion:* The JAK2V617F detection assay described here will contribute to early diagnosis of PV, ET and IMF because of its high sensitivity. In addition, quantification of JAK2V617F may contribute to disease management, especially when JAK2-specific inhibitors have become available for therapeutic use.

*Literature:* Baxter et al. Lancet 2005;365:1054. James et al. Nature 2005; 434:1144. Kralovics et al. N Engl J Med 2005; 352: 1779.

10.00 - 10.15 uur

**Verbetering van effciciënte hemocytometrische analyse door een combinatie van XE2100, SIS en DM96 (Sysmex)**

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*Inleiding:* Klinisch-chemische laboratoria streven naar efficiëntieverhoging. Kortere ligduur en ziekere patiënten leiden tot een veeleisende kliniek. Bovendien worden laboratoria geconfronteerd met bezuinigingsopdrachten. Personele kosten maken ruim 60% van de totale laboratoriumkosten uit. Een arbeids- en kennisintensief onderdeel van de diagnostiek is de beoordeling van perifere bloeduitstrijken. Zelfs de huidige generatie hemocytometrieapparatuur is onvoldoende in staat het aanbod adequaat te kunnen beoordelen. Door toepassing van geavanceerde automatiseringstechnieken binnen de hemocytometrie zijn wij in staat gebleken de personele inzet sterk te reduceren.
*Methode:* Op het GKCL wordt voor hemocytometrische analyse gebruik gemaakt van Sysmex apparatuur (Goffin-Meyvis). Middels het SIS (Sysmex Information System) kunnen resultaten van de XE2100's en de XT1800 automatisch gevalideerd worden op basis van vastgelegde beslisregels, voorgaande resultaten en additionele criteria. Door introductie van de DM96 kunnen perifere bloeduitstrijken middels een digitaal microscoopsysteem gecombineerd met beeldanalysesoftware automatisch beoordeeld worden. De toepassing van de combinatie van de systemen is uitgebreid geëvalueerd.
*Resultaat:* Voor introductie van het SIS diende circa 29% van de aangeboden monsters manueel beoordeeld te worden. Introductie van het SIS leverde 30% reductie in manuele differentiaties op en een aanzienlijke reductie in technische controles (bv. pseudoagglutinatie). Materiaal dat volgens SIS-criteria alsnog manueel geanalyseerd moest worden, werd aan de DM96 aangeboden. Na evaluatie blijken de resultaten van de DM96 voor meer dan 90% overeen te stemmen met de uitkomsten van de manuele differentiatie. Bovendien leidde de introductie van de DM96 tot een verdere reductie van meer dan 50% noodzakelijke 'hands-on'tijd.
*Conclusie:* De combinatie van SIS-software en DM96-digitale microscopie leverde een reductie van 50% aan personele inzet en reduceerde het aantal manuele differentiaties tot circa 2% van het oorspronkelijke aanbod.

## Sessie 2 Bedrijfsvoering

10.15 - 10.30 uur

**'Computer based' training; kwaliteitsborging voor POCT**

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*Inleiding:* De afgelopen jaren is uitvoering van kwalitatief hoogstaande point-of-caretesten en de bedrijfsvoering hieromtrent steeds professioneler geworden. Het laboratorium is verantwoordelijk voor kwaliteitsborging rondom POC-testen. Dit betreft niet alleen validatie van meter en gebruikte methode en uitvoeren van kwaliteitscontroles, maar ook training van gebruikers zodat zij over de juiste kennis en kunde beschikken. Gebruikers worden daarom vooraf geïnstrueerd in het gebruik van de meter, uitvoeren van kwaliteitscontroles, limitaties/interferenties en logistiek rondom POCT. Het blijkt echter dat deze kennis toch wegzakt met als gevolg mogelijk onbetrouwbare testresultaten. Omdat herhalingtrainingen door ploegendiensten en grote aantallen gebruikers logistiek moeilijk te organiseren zijn, hebben wij een 'computer based' training ontwikkeld welke leidt tot hercertificering van gebruikers.
*Methode:* Aan de hand van diverse aspecten van POCT-glucosemetingen werd een set van 150 multiplechoicevragen samengesteld. Deze vragenset werd beoordeeld door experts op gebied van POCT en kwaliteitsborging. Hierbij werd gestreefd naar toepasbaarheid van de training in elk ziekenhuis met een geprofessionaliseerd POCT-systeem met Accu Check Inform glucosemeters (Roche). Het resultaat werd omgezet in een 'computer based' training.
*Resultaat:* De 'computer based' training geeft een gebruiker aan de hand van een voortoets een studieadvies. De daarin genoemde onderdelen kunnen worden doorlopen waarbij gebruik gemaakt wordt van instructiefilmpjes, verschillende vraagmodellen (foto's, multiple choice, volgorde vragen, aanwijsvragen etc.) en uitleg. Zodra de gebruiker voldoende op de hoogte is wordt een examen afgelegd. Bij voldoende resultaat volgt hercertificering. Het programma is web'based' en kan daardoor op elke willekeurige plek en tijdstip worden gevolgd.
*Conclusie:* Om kwaliteitsborging van POCT te garanderen dienen gebruikers regelmatig te worden bijgeschoold. Gezien de organisatorische problemen bij herhalingstrainingen is een 'computer based' training hiervoor een uitstekende oplossing.

## Sessie 3 Klinisch

9.00 - 9.15 uur

### Increased levels of alpha-aminoadipic semialdehyde in body fluids from patients with pyridoxine-dependent epilepsy

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*Introduction:* Increased levels of L-pipecolic acid in cerebrospinal fluid (CSF) from patients with pyridoxine-dependent epilepsy (PDE) have been reported twice, and can be considered as a biomarker for this disease (1,2). Unfortunately, the increase of L-pipecolic acid is most pronounced in CSF and only modest in plasma, necessitating a lumbar punction for the collection of the CSF. Recent developments in the molecular and biochemical elucidation of PDE revealed that PDE is caused by an impaired L-pipecolic acid degradation at the level of alpha-amino adipic semialdehyde (AASA).
*Methods:* We investigated the levels of AASA in CSF, plasma and urine samples derived from PDE patients (most of them while on pyridoxine treatment) using liquid chromatography-tandem mass spectrometry.
*Results:* In body fluids derived from patients with PDE we found clearly increased levels of AASA: urine (n=11) 7 - 168 µmol/mmol creatinine (controls <1), plasma (n=8) 1.5 – 7.3 µmol/L (controls < 0.2), and CSF (n=7) 0.5 – 28 µmol/L (controls < 0.1).
*Conclusion:* The observation of increased levels of AASA in body fluids derived from PDE patients, yielded the biochemical evidence that PDE is caused by a defect in AASA dehydrogenase, resulting in an impaired conversion of AASA to AAA and the measurement of AASA can be used as a novel non-invasive biomarker for PDE, obviating the need of an inconvenient lumbar puncture, or a pyridoxine withdrawal.

*Literature:* 1. Plecko et al. Ann Neurol 2000; 48: 121-125.
2. Plecko et al. Neuropediatrics 2005; 36: 200-205.

9.15 - 9.30 uur

### Resolution of the enzymatic and molecular basis of 3-Hydroxyisobutyryl-CoA hydrolase deficiency, a new defect in valine oxidation

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*Departments of Clinical Chemistry[1] and Pediatrics[2], Emma Children's Hospital, Academic Medical Centre, Amsterdam, The Netherlands; Genetics Unit[3], Department of Biochemistry, University of Oxford, Oxford, UK; Royal Children's Hospital[4], Melbourne, Australia; Department of Biochemistry and Molecular Biology[5], Indiana University School of Medicine, Indianapolis, USA*

*Introduction:* Mitochondrial degradation of branched chain amino acids is initiated by transamination followed by decarboxylation resulting in branched chain acyl-CoA thioesters. Next, these thioesters are metabolized in a series of steps in which all the intermediates are CoA esters, with the exception for valine degradation. In the generally accepted view of valine breakdown, the portion of the pathway between 3-hydroxyisobutyryl-CoA and propionyl-CoA proceeds via free acids rather than the thioesters. Hence, a specific hydrolase is necessary to hydrolyze 3-hydroxyisobutyryl-CoA to 3-hydroxyisobutyryate and free CoA. In 1982 Brown and co-workers reported a patient manifesting a number of dysmorphic facial features, feeding difficulties, failure to thrive or develop motor skills (1). The authors found a marked reduction of hydrolase activity in the patient's fibroblasts. Our objective was to resolve the molecular basis of 3-hydroxyisobutyryl-CoA hydrolase deficiency.
*Methods:* We reinvestigated the 3-hydroxyisobutyryl-CoA hydrolase activity in fibroblasts and detected no release of CoASH in our patient. Next, anti-bodies raised against 3-hydroxyisobutyryl-CoA hydrolase from rat liver revealed the absence of this protein in the patient's fibroblasts.
*Results:* Mutation analysis of HIBCH on genomic DNA revealed a mutation in intron 3 (IVS3-9T>G) leaving the splice-site consensus unaffected. However, this mutation introduces an alternative cryptic splice acceptor site which is preferred over the authentic splice acceptor site, resulting in the retention of 8 intronic base pairs into the transcript causing a frame shift.
*Conclusion:* We provide conclusive evidence that mutations in HIBCH cause 3-hydroxyisobutyryl-CoA hydrolase deficiency and that in human part of valine catabolism proceeds via free acids, in contrast to the degradation pathway of leucine and isoleucine.

*Literature:* Brown et al. Pediatrics 1982; 70: 532-8

9.30 - 9.45 uur

**Acyl CoA dehydrogenase-9 (ACAD-9) is the long-chain acyl CoA dehydrogenase in human embryonic and fetal brain**

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*Introduction:* We recently reported the expression and activity of several fatty acid oxidation enzymes in human embryonic and fetal tissues including brain and spinal cord. Liver and heart showed abundant expression of both Very Long-Chain Acyl-CoA Dehydrogenase (VLCAD) and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) mRNA. In contrast, not only LCHAD activity but also acyl-CoA dehydrogenase (ACAD) activity was detected with palmitoyl-CoA as substrate in fetal central nervous tissue. These conflicting data suggest the active presence of a different long-chain ACAD in embryonic and fetal brain.
*Methods:* In situ, hybridization analysis was performed as described elsewhere (Oey et al. (2005)) (1). Acyl-CoA dehydrogenase activaties were measured enzymatically using a HPLC-based method described in Oey et al. (2005) (2).
*Results:* In this study, using in situ hybridization as well as enzymatic studies, we identified Acyl CoA dehydrogenase 9 (ACAD 9) as the long-chain ACAD in human embryonic and fetal brain and central nervous tissue.
*Conclusion:* The finding that ACAD-9 is the predominant, if not exclusive, acyl-CoA dehydrogenase in human embryonic and fetal brain is of utmost importance and may warrant screening for ACAD-9 deficiency in patients with unidentified neurological symptoms or neurological developmental disorders.

*Literature:* 1. Oey et al. Pediatr Res 2005; 57: 755-9. 2. Oey et al. J Inherit Metab Dis 2003; 26: 385-92.

9.45 - 10.00 uur

**Novel molecular defect in the platelet ADP receptor P2Y12 in a patient with a history of unexplained severe congenital bleeding**

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*Department of Clinical Chemistry and Laboratory Medicine[1], Department of Internal Medicine[2], Isala klinieken, Zwolle; Thrombosis and Haemostasis Laboratory[3], University Medical Centre, Utrecht, The Netherlands*

*Introduction:* The ADP receptor P2Y12 plays a crucial role in platelet activation and stable thrombus formation under flow conditions. Although intensive research is performed concerning the ADP receptors, only four unrelated patients with a molecular defect in the P2Y12 gene have been described so far. In the present study we performed platelet function studies to find an explanation for the severe bleeding tendency of a patient with an history of congenital bleeding.
*Methods:* 1) Platelet aggregation; 2) Perfusion studies; in which patient/control blood was perfused over collagen surfaces at arterial shear rates; thrombus morphology was analysed by microscopy; 3) DNA sequence analysis of patient's DNA.
*Results:* Impaired ADP and collagen induced platelet aggregation was observed, suggesting thrombocytopathy by secretion defects. Storage pool deficiency and defects in arachidonate metabolism were excluded and we suspected the patient to have a defective ADP receptor or defective ADP dependent signalling pathways. Subsequently, we studied thrombus morphology after perfusion of patient's blood over collagen. Patient's thrombi showed identical morphology compared to control formed in the presence of a P2Y12 antagonist. These results confirmed the hypothesis that our patient was likely to have defects in P2Y12. Therefore, we performed DNA sequence analysis of the P2Y12 gene and found a novel heterozygous base pair substitution C-to-A at nucleotide 1016 in exon 3, changing codon 258 from proline to threonine.
*Conclusion:* We present the first Dutch patient (fifth patient world-wide) with a novel molecular defect in the P2Y12 gene. An history of congenital bleeding is elucidated for our patient and his family. The attribution of perfusion studies revealed to be useful in the diagnostic process leading to sequencing patient's DNA for defects in P2Y12.

10.00 - 10.15 uur

**Inosine triphosphate pyrophoshohydrolase defiency: no longer a benign genetic trait?**

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*Introduction:* Inosine triphosphate pyrophosphohydrolase (ITPase) catalyses the pyrophosphohydrolysis of ITP and dITP to IMP and dIMP, respectively, generating inorganic pyrophoshate. Deficiency of ITPase activity causes intracellular accumulation of high levels of ITP. Recently, evidence was published that polymorphisms in the gene encoding ITPase are associated with adverse drug reactions to azathioprine therapy (1). These findings were confirmed in a prospective study which showed a significant correlation between the most common polymorphism in the ITPA gene ITPA 94C>A and azathioprine induced toxicity in both carriers and homozygous deficient individuals (2). We developed a sensitive method to determine ITPase activity and established reference values.
*Methods:* The pyrophosphohydrolysis of ITP in lysates prepared from peripheral erythrocytes was measured using ion-pair reversed phase HPLC.
*Results:* Reference values were established. In a patient referred because of azathioprine related toxicity (increased liver enzymes) and with a normal activity of thiopurine methyltransferase, we identified the first Dutch patient with a complete ITPase deficiency. Mutation analysis showed that this patient was homozygous for the ITPA 94C>A polymorphism.
*Conclusion:* We have developed a fast and sensitive assay to measure the ITPase activity in peripheral erythrocytes. We were able to identify a patient with an absent ITPase activity. Moreover, the absence of ITPase activity may provide an explanation for the liver toxicity observed in this patient. In ITPase deficient individuals treated with mercaptopurines an accumulation of 6-thio-ITP is expected, which is likely to cause cellular toxicity. We recommend measurement of both ITPase and TPMT activity for every patient who needs to be treated with mercaptopurine drugs.

*Literature:* 1) Marinaki A, et al. Pharmacogenetics 2004; 14: 181-7. 2) von Ahsen N, et al. Clin Chem 2005;52:2282-8.

10.15 - 10.30 uur

**Profiling the humoral immune response in colon cancer patients: diagnostic antigens from Streptococcus bovis**

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*Introduction:* The human bowel contains a large and dynamic bacterial population that is essential for intestinal health, but also critical for the development of diseases such as cancer. In this respect, the Gram-positive bacterium Streptococcus bovis has been associated with colon cancer for many years.
*Methods:* To investigate the clinical importance of this association, an immuno-capture mass spectrometry assay was developed that can generate infection-related protein profiles. The composition of these profiles is governed by the capture of specific antigens by serum antibodies from colon cancer patients.
*Results:* This assay showed that S. bovis antigen profiles could distinguish 11 out of 12 colon cancer patients from 8 control subjects, whereas antigen profiles derived from the gut bacterium Escherichia coli were not diagnostic for colon cancer. Moreover, S. bovis antigen profiles were also detected in polyp patients, indicating that infection with this bacterium does occur early during carcinogenesis. Highly accurate tandem mass spectrometry was used to identify one of the diagnostic antigens as a surface-exposed heparin-binding protein, which might be involved in attachment of S. bovis to tumor cells.
*Conclusion:* Together, these findings corroborate the hypothesis that colonic lesions provide a specific niche for S. bovis, resulting in tumor-associated 'silent' infections. These infections, however, only become apparent in colon cancer patients with a compromised immune system (bacteremia) or coincidental cardiac valve lesions (endocarditis). This makes profiling of the humoral immune response against 'silent' S. bovis infections a promising diagnostic tool for the early detection of human colon cancer, which is crucial for the effective treatment of this disease.