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Pharmacogenetics of severe 5-Fluorouracil toxicity

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5-Fluorouracil (5-FU) has been among the most commonly prescribed chemotherapeutic agents for the treatment of solid cancers for over 40 years. A high variation is observed in both tumor response and side effects to this drug, for which a genetic basis is suggested. So far, most pharmacogenetic studies have focused on polymorphisms in single genes; however, results have not been consistently reproducible, indicating a polygenic basis of 5-FU response and toxicity. Here we report results from a study including more than 110 cancer patients treated with 5-FU investigating potential associations of polymorphisms in multiple genes involved in 5-FU metabolism and the folate pathway with severe toxicity following 5-FU administration. Single gene associations as well as interactions between genetic polymorphisms and therapy regimen were assessed. Whereas no significant associations of single gene variants with severe 5-FU toxicity were observed, therapy-related factors, especially the co-administration of platinum compounds, were found to significantly influence the risk of 5-FU toxicity. However, combining both genetic data from multiple genes and therapy-related information significantly increased the correct classification of patients with severe toxicity as compared to either factor alone. Our data thus suggest that single polymorphisms cannot account for severe 5-FU toxicity alone and that variation in chemotherapy regimen is a potentially important confounder in pharmacogenetic studies. Nevertheless, the combined ge-

netic information from multiple genes was shown to significantly improve the prediction of severe 5-FU toxicity, when properly taking into account the therapy-related heterogeneity.

Population genetics of BH₄-responsive phenylketonuria (PKU)

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Mutations in the phenylalanine hydroxylase (PAH) gene result in phenylketonuria (PKU). Tetrahydrobiopterin (BH₄)-responsive hyperphenylalaninemia has been recently described as a variant of PAH deficiency caused by specific mutations in the PAH gene. It has been suggested that BH₄-responsiveness may be predicted from the corresponding genotypes. Data from BH₄ loading tests indicated an incidence of BH₄-responsiveness of >40% in the general PKU population and >80% in mild PKU patients. The current project entailed genotype analysis of 315 BH₄-responsive patients tabulated in the BIOPKUdb database and comparison with the data from the PAHdb locus-specific knowledgebase, as well as with previously published PAH mutations for several European countries, Northern China, and South Korea. We identified 60 mutations presenting with a substantial residual PAH activity (~47%), presumed to be associated with BH₄-responsiveness. More than 89% of patients were found to be compound heterozygotes. The three most common mutations found in >5% of BH₄-responsive patients were p.A403V,

p.R261Q, and p.Y414C. Using the Hardy-Weinberg formula the predicted average frequency of BH₄-responsiveness in European populations was calculated to be 55% (range 17–79%, lowest in Baltic countries and Poland and highest in Spain), 57% in Northern China, and 55% for South Korea. The genotype-predicted prevalence of BH₄-responsiveness was higher than prevalence data obtained from BH₄ loading tests. Inconsistent results were observed for mutations p.L48S, p.I65T, p.R158Q, p.R261Q, and p.Y414C. Our data suggest that BH₄-responsiveness may be more common than assumed and it may be predicted or excluded from the patient's genotype.

Phenotypic variability of BH₄ cofactor deficiency by intronic mutations in the PTS gene that lead to activation of distal splice sites and recruitment of pseudo-exons

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Tetrahydrobiopterin (BH₄) cofactor deficiency leads to an autosomal recessive inherited form of hyperphenylalaninemia in mild cases, but is mostly accompanied by a deficiency of the neurotransmitters dopamine and serotonin, termed severe cases. Thus far, over 250 patients with different mutations in the BH₄-biosynthetic gene PTS were described with various degrees of phenotypic severity (Thöny and Blau, Hum Mut 27:870–878, 2006). Here we report on two independent patients with distinct phenotypic manifestation of BH₄ deficiency due to intronic mutations causing aberrant splicing of the PTS pre-mRNA and recruitment of additional, cryptic exons as investigated in der-

mal fibroblasts. One patient with a mild phenotype had a homozygously inherited A to T transition in intron 1 in the polypyrimidine tract of the 3' splice site upstream of a cryptic exon 1a leading to erroneous recruitment of this 79 bp pseudo-exon between exons 1 and 2. Although mutant *PTS*-mRNA was predominant, wild-type transcript was also present which must be responsible for the mild phenotype. A second patient had a severe phenotype and was compound heterozygote for *PTS* with a paternal null allele due to 1 bp deletion in exon 6, and a maternal allele with a deletion of 57 bp in intron 2 upstream of a cryptic 45 bp-exon 2a. Transcript analysis showed no wild-type *PTS*-mRNA but two mutant species, a transcript with the 1 bp-deletion and an erroneously spliced *Pts*-mRNA containing the pseudo-exon 2a, indicating an aberrant splicing with an unusual high penetrance. In conclusion, intronic mutations in the *PTS* gene which can activate to various degrees pre-existing pseudo splice sites distal from the mutation, thereby recruiting cryptic exons, can influence the severity of BH₄-cofactor deficiency. (Supported by SNF)

Correction of murine PKU following AAV-mediated intramuscular expression of a complete phenylalanine hydroxylating system

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Phenylketonuria (PKU) due to hepatic phenylalanine hydroxylase (PAH) deficiency leads to systemic accumulation of phenylalanine (Phe), associated with mental retardation, microcephaly, and hypopigmentation. The PAH deficiency has no direct pathologic effect upon the liver itself but rather chronically elevated Phe damages the developing brain. Previously, we reported long-term correction of hyperphenylalaninemia in our PKU mouse model following liver directed gene transfer with a recombinant adeno-associated virus (rAAV2) pseudotype 8 vector

(Ding, Georgiev and Thöny, *Gene Ther* 13:587–593, 2006). However, questions of expression stability, treatment toxicity, potential for insertional mutagenesis, and safety of the invasive techniques required to access human liver remain unanswered for this approach. PAH is predominantly expressed in liver and its activity requires a supply of tetrahydrobiopterin (BH₄) cofactor, but we propose that expression of a complete phenylalanine hydroxylating system (PAH plus BH₄ synthetic enzymes) in skeletal muscle will lead to therapeutic reduction of blood Phe levels in PKU mice. To test this hypothesis, we first developed transgenic PKU mice that lack liver PAH activity but co-express in skeletal muscle PAH and GTPCH, the latter responsible for the committing enzymatic step in BH₄ biosynthesis. Despite sufficient muscle enzyme expression, these mice remained hyperphenylalaninemic suggesting that expression of additional BH₄ synthetic enzymes would be necessary. A recombinant triple-cistronic rAAV2 pseudotype 1 vector expressing PAH along with GTPCH and PTPS, the next step in BH₄ synthesis, was generated. Injection of this vector into gastrocnemius muscles of PKU mice led to stable and long-term reduction of blood Phe and reversal of PKU-associated coat hypopigmentation. We propose that muscle-directed gene therapy will be a viable alternative treatment approach to PKU and other inborn errors of metabolism. Supported by SNF.

Clinical, biochemical and molecular genetic analysis in a patient with a severe myopathy

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The mitochondrial tRNA mutation A3302G leads to abnormal mitochondrial RNA processing and is associated with adult onset progressive myopathy and cardiorespiratory problems. We will present a patient with this tRNALEU(UUR) mutation with an early onset, severe phenotype. The patient was first investigated at the age of 4 years because of increased fatigabil-

ity since two years, but otherwise normal psychomotor development and normal cardiac function. At rest there was a generalized moderate hypotonia but after a short activity strong fatigability and loss of head control occurred. The disease progressed rapidly to a severe myopathy with respiratory involvement. Investigations: lactic acidosis, elevated CK and paradoxical ketosis were present; cerebral MRI/MRS was normal. Muscle biopsy showed ragged red fibers and analysis of the respiratory chain complexes showed a 10% residual complex I (CI) activity. Analysis of the mitochondrial genome from skeletal muscle revealed a homoplasmic A3302G transition in the tRNA. Fibroblast CI activity was only slightly below the control range, which correlated with the respirometric findings. This mutation is usually associated with a juvenile or adulthood onset, slowly progressive myopathy. This patient enlarges the clinical spectrum of this mutation, being the youngest patient described with a severe phenotype. Similarly to the other published cases, this mutation affects CI activity in skeletal muscle but not in fibroblasts. The severity of the phenotype may be related to the mutation load of the affected tissue. This patient widens the clinical spectrum caused by this specific mt tRNA mutation, with important consequences for genetic counselling in affected families.

Multiple OXPHOS deficiency in the liver of a patient with homozygous MTDNA polymerase gamma mutation (A467T) and heterozygous thymidine phosphorylase mutation (A465T)

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Mutations in mitochondrial DNA polymerase gamma (POLG) produce a broad clinical spectrum. Childhood presentations do have a clear male predominance and, interestingly, patients with a homozygous A467T mutation seem to have a relatively mild clinical course. We will present a

12 year old female patient with valproate-induced liver failure, liver transplantation and subsequent severe clinical deterioration. The patient with normal development received valproate for generalized seizures. After 4 months she developed acute liver failure (ASAT 274U/L, ALAT 389U/L, NH3 219umol/l, factor V 33%, INR 2.0) and underwent liver transplantation. After 1 week, she had a stroke with diffuse lesions occipital, frontal and in the thalamus. She developed continuous generalized myoclonus, neuropathy, cortical blindness and intestinal pseudo-obstructions needing continuous feedings. Investigations revealed persistent elevated lactate. Respiratory chain complex I, III, and IV activities were 8%, 50% and 20%, respectively in liver. Activities in muscle and fibroblasts were normal. Mutational analysis of POLG showed a homozygous A467T mutation and a heterozygous (A465T) mutation in the thymidine phosphorylase gene. Reported cases in childhood are mostly male. Patients with homozygous A467T mutation mainly develop encephalopathy, none received valproate and did not develop liver failure. Liver failure with encephalopathy seems a main characteristic in compound heterozygous patients. We hypothesise that the severe clinical course of our patient was due to valproate administration (environmental effect) and to the additional heterozygous mutation in the TP gene (a protein known to induce also mtDNA depletion) that may increase disease expression.

The dissociated expression of AGAT, GAMT and CT1 in CNS suggests the transport of guanidinoacetate between brain cells for creatine synthesis to occur

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The lack of creatine transporter (CT1) in astrocytes makes the import of creatine from blood inefficient in CNS, which relies more on endogenous creatine synthesis through AGAT and GAMT expression. This seems contradictory with CT1 deficiency, which, despite AGAT and GAMT expression, leads to creatine lack in CNS. To elucidate this, our aim was to finely dissect

the cell-to-cell (co-)expression of AGAT, GAMT and CT1 in CNS. AGAT, GAMT and CT1 (co-)expression was analyzed by combining in situ hybridization and immunohistochemistry. The proportions of cells expressing AGAT, GAMT, CT1, AGAT+GAMT, AGAT+CT1, GAMT+CT1, AGAT+GAMT+CT1, or none, were calculated in various regions of the rat brain (cortex, caudate putamen, hippocampus, hypothalamus, inferior colliculus, pons, cerebellum). In most structures, cells co-expressing AGAT+GAMT, equipped to self-synthesize creatine, were <20%. Cells co-expressing GAMT+CT1 were also <20%. In whole CNS, 30–50% of cells did not express AGAT nor GAMT, and only 2–15% expressed CT1 alone. In cortex and caudate putamen, very few cells seemed able of their own creatine synthesis, in agreement with the creatine lack observed by MRS in CT1-deficient patients. Our work suggests that to allow CNS synthesis of creatine, guanidinoacetate must be transported from AGAT- to GAMT-expressing cells possibly through CT1, thus explaining why CT1-deficient patients lack creatine in CNS. Moreover, high proportion of cells with no expression of AGAT, GAMT and CT1, and low proportion of cells expressing CT1 alone, suggest that brain cells express AGAT, GAMT and CT1 on demand to timely adapt their creatine needs.

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The blood/brain barrier in neonates: 1H-MR spectroscopy shows low Protection against high Phenylalanine

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Background: ¹H-MRS has been used to determine the blood/brain ratio of Phenylalanine (Phe) in adult patients with phenylketonuria (PKU). While the exact value is still debated, there is consensus that brain Phe is 2–4 times lower than in blood. The most crucial time for outcome is early childhood but data on the blood/brain ratio of Phe in newborns are not available. **Objective:** To determine the blood/

brain ratio of Phe in neonates with PKU.

Methods: All spectra were recorded on a 1,5 TMR scanner. So far, 2 neonates with PKU were investigated: Patient A, 43 weeks gestational age (GA), at 9 days; Patient B 36 weeks GA at 9 and 14 days. Controls: 2 healthy neonates 43 and 44 weeks GA. Adults: 6 PKU patients (23±8y old) and 6 healthy subjects.

Results: ¹H-MRS spectra were all of good quality. The blood/brain ratio was strikingly higher for neonates than adults. After treatment, Phe dropped to normal in parallel with blood levels in patient B.

PKU Patients	Blood Phe (mM)	Brain Phe (mmol/kg)	Ratio
Neonate A	0.75	0.39	0.52
Neonate B	0.29	0.22	0.77
Neonate B	0.05	0.07	(1.2)
6 Adults	0.72±0.3	0.21±0.7	0.29±0.04

Discussion: This study shows that the blood brain barrier does not provide the same protection against high Phe for newborns as it does for the adults. At identical blood Phe levels the brain of newborn PKU patients is exposed to much higher Phe level than the brain of adults. This underlines the importance of strictest dietary control in young age.

Normalisation of odd-numbered long-chain fatty acids and severe cardiomyopathy following liver transplantation in propionic acidemia

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Background: Propionic acidemia (PA) is caused by a deficiency of propionyl-CoA carboxylase. The accumulation of propionyl-CoA leads to organic aciduria and enhanced synthesis of odd-numbered long-chain fatty acids (OLCFA) stored in adipose tissue. As the liver is responsible for the metabolism of many of the precursor compounds, orthotopic liver transplantation (OLT) has been described as a treatment option. Furthermore, OLT has been suggested to stabilize cardiomyopathy, a frequent long term complication in PA.

Objective: To present the clinical outcome and changes in OLCFA stores in adipose tissue and other characteristic biochemical parameters before and after OLT in a patient with PA.

Patient: Our patient with late onset PA and hitherto mild clinical course developed a rapidly progressive cardiomyopathy at the age of eighteen.

Methods: Fatty acid methyl esters were analysed by GC-MS with a stable isotope dilution method. The sum of the OLCFA (C15:0, C17:0) was expressed as percentage of total fatty acids (C14:0–C18:2). Propionate and organic acids were analysed by standard methods

Results: We found a normalization of characteristic parameters 3 years after OLT: OLCFA (3.7% versus <1%), propionate (16.6 $\mu\text{mol/l}$ versus none), methylcitrate (0.16 mol/mol creatinine versus none). Interestingly, in the same time we observed a reversal of cardiomyopathy: ejection fraction (20% versus 55%), maximal performance capacity (111 Watt versus 147 Watt).

Discussion: OLT may normalize characteristic parameters of PA measured in urine and blood and in the long run also OLCFA in adipose tissue. Obviously, OLT does not correct the defect in other tissues. We hypothesize that the beneficial effect of OLT on cardiomyopathy is mainly due to reduction of OLCFA and propionate (inhibitory metabolite) after transplantation.

New method for the determination of free and total carnitine by electrospray tandem mass spectrometry

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The interpretation of the acylcarnitine patterns in blood, as measured by tandem mass spectrometry could be hampered by several side-reactions taking place during the derivatization step of the method. There is a time course- and a temperature-dependant hydrolysis of the acylcarnitines which relies on the length of the acyl moiety of the compounds. Therefore, to quantitate reliably the free as well as the acyl carnitine fractions (which are both part of the carnitine status), we developed a new method for the measurement of non-derivatized free and

total carnitine in blood and urine by isotope dilution electrospray tandem mass spectrometry. In this method plasma or urine are mixed with carnitine-D3. For total carnitine determination, the acylcarnitines were hydrolyzed in the presence of KOH. Carnitine and carnitine-D3 were purified by micro-SPE with an overall yield of 59 %. Separation of the analytes was achieved at 30 °C using an Atlantis HILIC silica column. The column eluent was monitored using a triple quadrupole mass spectrometer. The tandem mass spectrometer was programmed using the selected reaction monitoring mode (SRM) at m/z 162 to 103 for carnitine and at m/z 165 to 103 for carnitine-D3. We obtained an inter-day assay coefficient of variation respectively of 7.1 % and 4.8 % at 35 $\mu\text{mol/l}$ and 103 $\mu\text{mol/l}$ of carnitine. The proposed method by isotope dilution electrospray was validated by comparison with the reference radio-enzymatic assay. *In conclusion*, the accurate quantification of free and total carnitine in plasma and urine was achieved using SPE and tandem mass spectrometry. This method provides high repeatability and is a reliable alternative for replacing the radio-enzymatic assay which is laborious and time consuming.

Selective screening of purine and pyrimidine errors of metabolism by liquid chromatography coupled with tandem mass spectrometry

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Analysis of purines and pyrimidines by HPLC or capillary electrophoresis (CE) used to selectively screen the disorders of purine and pyrimidine metabolism are prone to numerous interferences. Therefore a LC-MS/MS method for purine and pyrimidine metabolites was developed for urine samples. Urine is diluted to a creatinine concentration of 1 mmol/l and mixed with stable isotopes of labeled internal standards for the quantification. Purines and pyrimidines are extracted with methanol using a blotting paper (Wattman n°903) and separated within 23 min using an Aqua C18 column (Phenomenex). The selected reaction monitoring mode (SRM) al-

lows to produce a calibration curve and quantify 25 metabolites. Most of them are detected by using a negative electrospray ionization (ESI). The assay was validated using spiked pooled urines as matrices. The overall recovery of the substances are within 68 and 100% at the exception of 2,8-DHA and succinyladenosine (40 and 35% respectively). There is a suppressive matrix effect within 0–32% at the exception of 5-hydroxymethyluracil (45%). MS analysis of the urine revealed less interference and better sensitivity than the CE method. Depending on the metabolite, limit of quantification (LOQ) is between 0.1 and 6 mmol/L. In addition to the uric acid and amino acids quantification in urine, this LC-MS/MS method should enable the detection of purine and pyrimidine metabolism defects with high specificity and sensitivity.

Changes of coagulation parameters during high altitude expedition

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Aim: Data on changes of hemostatic parameters at altitudes above 5000 m are very limited. So far it is unknown, whether altered coagulation could contribute to the development of acute mountain sickness.

Methods: 34 healthy mountaineers were randomized to 2 acclimatization protocols and underwent an expedition on Muztagh Ata (7549m). Tests were performed at 5 altitudes: Zurich pre-expedition (PE,450m), Basecamp (BC,4497m), Camp1 (C1,5533m), Camp2 (C2,6265m), and Camp3 (C3,6865m). The following parameters were assessed together with Lake Louise AMS score: PT, aPTT (global tests), D-Dimer (activation marker), APC-resistance, von Willebrand Factor activity (RCo) & ADAMTS-13 & C-natriuretic peptide (CNP).

Results: D-Dimer increased with altitude (0.62 to 0.81 $\mu\text{g/L}$, $p < 0.0001$). During ascent, PT increased (83% to >100%) and APCR decreased from 0.95 to 0.8 (both $p < 0.01$). An increase of aPTT (38 to 43 sec) was paralleled by changes of RCo (102% to 62%) (both $p < 0.001$), possibly indicating increased consumption of von Willebrand Factor. Interestingly, moun-

taineers reaching the mountain peak had lower D-Dimer concentrations ($p < 0.01$), longer aPTT ($p = 0.06$) and lower ADAMTS-13 activity ($p < 0.01$) than mountaineers, who had to stop ascent due to different reasons. None of the parameters showed a relationship with AMS scores. When comparing the participants of the two acclimatization protocols, there was a higher RCo activity in patients with faster ascent protocol ($p = 0.04$) accompanied by lower ADAMTS-13 activity ($p = 0.04$).

Conclusions: Coagulation parameters change during high altitude expedition. Whereas we could not detect any association of AMS scores and coagulation parameters, our results exhibit some parameters to be associated with acclimatization protocol and successful ascent to Muztagh Ata Peak.

Glomerular filtration rate estimates decrease during high altitude expedition but increase with Lake Louise Acute Mountain Sickness Scores

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Aim: Acute mountain sickness (AMS) can result in pulmonary and cerebral edema with overperfusion of microvascular beds, elevated hydrostatic capillary pressure, capillary leakage, and consequent edema as pathogenetic mechanisms. Data on changes in glomerular filtration rate (GFR) at altitudes above 5000 m are very limited.

Methods: 34 healthy mountaineers were randomized to 2 acclimatization protocols and underwent an expedition on Muztagh Ata (7549 m) in China. Tests were performed at 5 altitudes: Zurich pre-expedition (PE, 450 m), Basecamp (BC, 4497 m), Camp1 (C1, 5533 m), Camp2 (C2, 6265 m), and Camp3 (C3, 6865 m). Cystatin C-based and creatinine-based (Mayo Clinic quadratic equation) GFR estimates (eGFR) were assessed together with Lake Louise AMS score and other tests.

Results: eGFR significantly decreased from PE to BC ($p < 0.01$). However, when looking at changes between BC and C3, only cystatin C-based estimates indicated a significant decrease of GFR ($p = 0.02$). There was a linear decrease of eGFR from PE to C3, with

a decrease of about 3.1 ml/min/1.73m² per 1000 meters increase of altitude. No differences between eGFR regarding acclimatization protocol could be observed. There was a significant association between eGFR and hematocrit ($p = 0.01$), whereas no significant association between eGFR and aldosterone, renin, and BNP could be observed. Finally, higher AMS scores were significantly associated with higher eGFR ($p = 0.01$).

Conclusions: Renal function declines when ascending from low to high altitude. Cystatin C-based eGFR decreases during ascent in high altitude expedition but increase with AMS scores. For individuals with eGFR < 40 ml/min/1.73m², caution may be necessary when planning trips to high altitude above 4500 meters above sea level.

Decreased kidney function and the coronary angiographic state are mutually independent predictors of future vascular events

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Background: Data on the relationship between kidney function markers, angiographic state and future cardiovascular events in coronary patients are scarce.

Methods: We assessed kidney function by means of several markers (cystatin C, cysC; beta-2-microglobulin, B2M; beta-trace protein, BTP; urea, Ur; creatinine, Cr; MDRD; Mayo Clinic Quadratic Equation, MCQE) in 195 consecutive patients (age 63±10 years; 30.3% female) undergoing coronary angiography for the evaluation of stable coronary artery disease (CAD). Prospectively, we recorded major coronary events and cumulative vascular events over 6 years.

Results: 61.5% of the patients had significant coronary stenoses with a lumen narrowing $\geq 50\%$. With the exception of Cr ($p = 0.002$), there were no significant differences among the different kidney function markers between patients with CAD and those in whom angiography did not show CAD. In logistic regression, adjusting for age, sex, BMI, diabetes mellitus, smoking, hypertension, CRP, LDL & HDL, none of the investigated kidney function pa-

rameters was associated with significant coronary stenoses. Prospectively, the risk of future vascular events, as assessed by Cox regression analysis was significantly increased with CysC, B2M, Ur, and Cr-based methods, independently from coronary angiographic status ($p < 0.05$ for all).

Conclusions: Among coronary patients, CysC, B2M, Ur and Cr-based methods to estimate GFR independently from coronary angiographic status predict future vascular events.

Moderate to severe chronic kidney disease is a very prevalent condition among elderly non-nephrological patients with anemia

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Background: Anemia represents one of the most often encountered symptoms in clinical practice. Whereas decreased production of erythropoietin in chronic kidney disease (CKD) is well known as a potential cause of anemia, the contribution of CKD to anemia has not yet fully been defined on a public health basis.

Methods: We therefore conducted a cross-sectional study consecutively enrolling adult patients from the principality of Liechtenstein seeking non-nephrological medical care, who were simultaneously investigated for anemia and renal function. The glomerular filtration rate (eGFR) was estimated by the MDRD formula; anemia was diagnosed according to WHO criteria.

Results: Data were available for 38% ($n = 8410$) of the country's entire population ≥ 25 years. Anemia was present in 15.7%. An eGFR indicating CKD stages 3–5 (< 60 ml/min/1.73m²) was found in 8.8%. Prevalence of an eGFR < 60 ml/min/1.73m² in anemic patients amounted to 27% overall and showed a significant increase with age: 1.4% at age 25–34, 4.9% at age 35–44, 9.6% at age 45–54, 17.6% at age 55–64, 26.5% at age 65–74, 37.6% at age 75–84 and 56.3% at age 85 and above ($p < 0.001$). The respective prevalences of eGFR < 30 ml/min/1.73m² were lower (7.4% overall) but still substantial: 0.7%, 2.5%, 3.8%, 6.8%, 9.5%, 9.7% and 10.4%

($p < 0.001$). In a logistic regression model, age (OR 1.036, 95%CI 1.032, 1.040 per year), female gender (OR 1.33, 95% CI 1.17, 1.52) and CKD stage 3–5 were significantly predicting the presence of anemia, with CKD stage 3–5 being the strongest predictor (OR 3.025, 95%CI 2.53,3.61). An interaction term age*eGFR category was highly significant ($p < 0.001$), indicating that the association between impaired renal function and anemia was significantly stronger among older patients than among younger individuals.

Conclusions: An eGFR $< 60 \text{ ml/min/1.73m}^2$ is a strong independent predictor for anemia, especially in elder patients. With a prevalence of about 50% in patients > 80 years, impaired renal function appears to be among the most important causes of anemia in this age group.

Serum concentrations of methyl malonic acid but not holotranscobalamin are affected by glomerular filtration rate

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Background: Vitamin B12 (B12) deficiency can e.g. be defined by increased methylmalonic acid (MMA) or decreased B12 concentrations. B12 is a convenient but insensitive marker of B12 deficiency. Recently, Holotranscobalamin (HoloTc) has been proposed as more sensitive marker already identifying early stages of B12 deficiency.

Methods: We conducted a cross-sectional study in 530 adult patients (58.7% female) with samples having referred for investigation of B12 deficiency. B12 was measured by Unicel (Beckman Coulter), HoloTc was assessed on AxSYM (Abbott Diagnostics), whereas MMA was determined by GC/MS. In order to capture an influence of renal function markers on the investigated parameters, ID-MS traceable creatinine was assessed by a kinetic Jaffe method.

Results: A total of 530 adult patients (58.7% female) were simultaneously investigated for all 3 markers of B12 deficiency. 7.4% had decreased B12 ($< 125 \text{ pmol/L}$), 75.5% had B12 in the grey zone (125–300 pmol/L), and

17.2% had normal or increased B12 levels. Of the patients with grey zone B12, 27.5% had decreased HoloTc values ($< 35 \text{ pmol/L}$) indicating B12 depletion, 20% had normal HoloTc values $> 50 \text{ pmol/L}$. Of the patients with grey zone or normal HoloTc, 8.6% had abnormal MMA ($> 271 \text{ nmol/L}$), 64% of which had at least mildly reduced eGFR $< 90 \text{ ml/min/1.73m}^2$. There was a significant inverse correlation between MMA and eGFR ($r = -0.295$, $p < 0.001$). No significant association could be observed between HoloTc and eGFR.

Conclusions: B12 deficiency is a prevalent condition, especially at early stages. HoloTc, in contrast to MMA, is not influenced by decreased kidney function. In order to define early stage B12 deficiency in patients with impaired GFR, the use of HoloTc rather than MMA is suggested.

Usefulness of active-B12 as a new marker for cobalamin deficiency

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Cobalamin (vitamin B12) deficiency is associated with haematological and neurological manifestations. The prevalence of this condition is particularly high in vegetarians and elderly people. Inappropriate intake and malabsorption of vitamin B12 are the main causes leading to this condition. The early identification of serum cobalamin depletion in the subclinical phase is an essential step in the prevention of irreversible neurological impairment. Therefore, early, sensitive and specific biochemical markers are needed. Recently, a new marker of cobalamin status, the holotranscobalamin (HoloTc or active-B12), has been introduced. Active-B12 corresponds to the metabolic active serum cobalamin and seems to fulfil these criteria.

Results: A retrospective analysis performed at the Kantonsspital Aarau showed 45% of total-B12 results are within a grey zone (between 150 and 300 pmol/l). Inpatients showing unclear neurological manifestations were assessed for vitamin B12 state (by total-B12 and active-B12). Active-B12 proved to be a useful biochemical marker for the discrimination of about 2/3 of total-B12 borderline results,

avoiding time consuming and expensive further investigations.

Discussion: So far no consensus has been found about the algorithm to be applied when performing laboratory diagnosis for cobalamin deficiency. Our results suggest the determination of active-B12 for the discrimination of borderline total-B12 results. Further, we propose a modified algorithm for workup of suspected vitamin B12 results.

Iodide in urine – an exciting challenge

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Background: Iodide is essential for the synthesis of thyroid hormones by the thyroid gland. Measurement of iodide in urine is therefore frequently used in investigations of thyroid dysfunction. Pathologies may be due to iodine deficiency or excessive iodine exposure. Iodine in urine is most frequently measured as total iodine based on the Sandell-Kolthoff reaction. This method however is time consuming and uses toxic and irritating reagents requiring special working conditions. Ion chromatography (IC) is an alternative method.

Objective: To evaluate the feasibility of iodide measurement in urine with an ion chromatography system (Dionex DX-600) and comparison with the Sandell-Kolthoff method.

Methods: IC coupled with electrochemical detection (constant applied potential, +0.85V versus Ag/AgCl) using a modified platinum (Pt) working electrode. Sample preparation includes centrifugation, filtering (0.45µm nylon membrane), dilution 1:5 with water and treatment with a polymeric reversed-phase cartridge (OnGuard™-RP). The Sandell-Kolthoff reaction is based on a Ce^{4+} reaction with As^{3+} ions in sulphuric acid, catalysed by iodide and photometric detection. This reaction is preceded by acid hydrolysis of the sample.

Results: The IC-EC detection method showed a good precision in the very low concentration range (25nmol/l: intra-assay CV 0.2% – 1.9%, inter-assay CV 4.5% – 7.2%), as well as at very high (1000nmol/l) iodide concen-

trations. With the modified Pt electrode we achieved a symmetrical peak, a stable base line which was not possible with an Ag-electrode. However, there was a progressive signal loss of about 20% after a 4.5 hours run. The comparison of the two methods with Passing Bablok analysis showed the following correlation: $y = 1.0771x - 31.076$.

Conclusions: The IC-EC detection method is a fast, non toxic, sensitive method for analysing iodide in urine. The analysis of large series is hampered by the progressive loss of signal intensity. Further investigations are in progress in order to obtain better signal stability.

Validation of the 25-OH vitamin D assay on the LIAISON automated analyzer

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Introduction: 25-OH vitamin D is a precursor of calcitriol, which is involved in the regulation of calcium and phosphate levels in the blood. Measurement of 25-OH vitamin D is used to assess the overall vitamin D status. Recently, an automated assay was introduced (Diasorin).

Methods: The performance of the new Liaison 25-OH Vitamin D assay was compared with a validated competitive RIA (25-OH vitamin D RIA, Immunodiagnostic Systems LTD). Imprecision was evaluated at 4 levels in duplicates over 10 days.

Results: 79 serum samples were analyzed by both methods and compared by Passing & Bablok regression: Liaison = $1.13 \cdot \text{RIA} - 6.9\text{nM}$. Interassay imprecision of the Liaison assay was 9.4%, 11.5% and 14.7% at 25-OH vitamin D concentrations of 53nM, 27nM and 18nM respectively. Functional sensitivity (interassay coefficient of variation of single measurements <20%) was 12nM. Dilution linearity yielded a regression equation of observed = $0.94 \cdot \text{expected} + 3.7\text{nM}$.

Conclusion: The determination of 25-OH vitamin D by the LIAISON automated system shows comparable results to a validated RIA method, and thus, provides a valid option for a non-radioactive assay.

Assessing sample stability of hematology analytes: Are there any differences between various hematology analyzers? A reference method study

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Aim: With the availability of novel hematology analyzers, novel measurement techniques are being introduced into daily routine. As analyte stability might differ according to the employed measurement technique, the aim of this study was to assess the stability of hematologic analytes on different hematology analyzers.

Methods: We investigated the effect of storage time and storage temperature on sample stability on three novel analytical systems (Advia 120, Bayer Diagnostics; XE 2100 Sysmex and LH 755 Beckman Coulter). Samples were obtained from 64 healthy volunteers and stored at room temperature as well as at 4–8°C in order to conduct analysis at different times up to 72h after blood was drawn. Sample stability was assessed by graphical truncated normal sequential test. A parameter was considered stable, when its average change was smaller than one CV (%) of the assessed method, allowing a 5% risk of error.

Results: Red blood cell counts and hemoglobin were least affected by storage temperature and showed stability for at least 48h, depending on the analyzer utilized. While reticulocytes, MCV, hematocrit and leukocyte counts were more stable at 4–8°C, thrombocytes exhibited a better stability at RT. The white blood cell (WBC) sub-populations changed over time with a decrease in eosinophils and lymphocytes and an increase in neutrophils at 4–8°C. Further, the stability patterns of WBC sub-population were significantly different among the 3 investigated analyzers with some analytes only displaying a stability of 4h.

Conclusions: Analyte stability of hematological parameters varies not only according to the investigated parameter but also according to storage temperature and the employed meas-

urement system. Depending on the analyte sample stability differences among the different analytical systems are considerable.

First results with a multiplex real-time PCR assay for detection of bacteria and fungi in blood

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Polymerase chain reaction (PCR) accelerates the diagnosis of disease causing agents and increases the analytic sensitivity. This argument applies even more to real time PCR (RT-PCR), which measures the increase and specificity of the PCR products during each PCR cycle. Post-PCR analysis of the PCR products is not necessary, which decreases further the time necessary to receive a result. Many PCR and RT-PCR methods are multiplex assays, which generate and analyze several PCR products in the same reaction. A commercial multiplex RT-PCR kit for the detection and identification has recently been launched (Septifast®). It can differentiate up to 30 different microbiological species relevant in the pathogenesis of sepsis. We set up a collaborative effort to evaluate the sensitivity, practicability, and cost effectiveness of the Septifast® assay, compared to microbiological analysis. Until now, 11 samples from intensive care patients were analyzed with the standard microbiological protocol (blood culture) and the Septifast® analysis. In 6 samples, both methods gave negative results. In two samples, the positive results were in agreement between the two methods. In three samples, there were divergent results: one sample tested positive in the Septifast® assay and negative in blood culture, in another sample it was vice versa (negative in the Septifast® assay and positive in blood culture). In one sample, the positive results of the two methods were different. The analysis of additional samples will give us a better picture of the sensitivity and specificity of the Septifast® assay, as well as estimates of cost efficiency and time requirements.