216. Molecular pathology and functional genomics of lung disease

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Late-breaking abstract: The TERT-CLPTM1L locus for lung cancer predisposes to bronchial obstruction and emphysema

Els Wauters[1,2,3], Dominiek Smeets[1,2], Johan Coolen[4], Johny Verschakelen[4], Paul De Leyn[5], Marc Decramer[3], Johan Vansteenkiste[3], Wim Janssens[3], Diether Lambrechts[1,2]. *[1]Vesalius Research Center (VRC), KULeuven, Leuven, Belgium; [2]Vesalius Research Center (VRC), VIB, Leuven, Belgium; [3]Respiratory Division, University Hospital Gasthuisberg, KULeuven, Leuven, Belgium; [4]Department of Radiology, University Hospital Gasthuisberg, KULeuven, Leuven, Belgium; [5]Department of Thoracic Surgery, University Hospital Gasthuisberg, KULeuven, Leuven, Belgium*

Clinical studies suggest that bronchial obstruction and emphysema increase susceptibility to lung cancer. We assess the possibility of a common genetic origin and investigate whether the lung cancer susceptibility locus on chromosome 5p15.33 increases the risk for bronchial obstruction and emphysema.
Three variants in the 5p15.33 locus encompassing the TERT and CLPTM1L genes were genotyped in 777 heavy smokers and 212 lung cancer patients. Participants underwent pulmonary function tests and computed tomography (CT) of the chest, and took questionnaires assessing smoking behaviour.
The rs31489 C-allele correlated with reduced forced expiratory volume in 1 second (FEV1; P=0.006). Homozygous carriers of the rs31489 C-allele exhibited increased susceptibility to bronchial obstruction with an odds ratio (OR) of 1.82 (95% confidence interval [CI]=1.24-2.69; P=0.002). A similar association was noticed for lung diffusing capacity (DLCO; P=0.004). Consistent herewith, CC-carriers had an increased risk of emphysema (OR=2.04; CI=1.41-2.94; P=1.73×10^{-4}) and displayed more alveolar destruction. Finally, CC-carriers also had an increased risk for lung cancer (OR=1.90; CI=1.21-2.99; P=0.005) and were more susceptible to develop both lung cancer and bronchial obstruction than lung cancer alone (OR=2.11; CI=1.04-4.26; P=0.038).
The rs31489 variant on 5p15.33 is associated with bronchial obstruction, the presence and severity of emphysema and lung cancer.

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Late-breaking abstract: Possible association between vitamin D receptor SNP FokI and treatment response in asthmatic children

Helly Einisman[1], María Loreto Reyes[1], Marcelo Lopez-Lastra[1], Jenniffer Angulo[2], Marie Solange Caussade[1], Ana Moya[1], Jaime Cerda[3], Jose A. Castro-Rodriguez[1]. *[1]Pediatrics, Pontificia Universidad Católica de Chile, Santiago, Chile; [2]Medical Investigation Center, Pontificia Universidad Católica, Santiago, Chile; [3]Public Health, Pontificia Universidad Católica, Santiago, Chile*

Introduction: An association between poor asthma control and low 25OH Vitamin D (25OHD) has been shown. Single nucleotide polymorphisms (SNP) in the gene encoding 25OHD receptor (VDR) have been associated with asthma. Hypothesis: asthma control is associated with 25OHD levels and VDR SNP.
Aims: To compare 25OHD levels in controlled and uncontrolled asthmatic children and healthy controls; to genotype, in asthmatics, 2 SNP in the VDR gene.
Methods: 25OHD values in healthy children were obtained from a sample of a previous study. Asthmatics treated with at least 3 months of inhaled steroids were enrolled in outpatient clinics. A clinical questionnaire of asthma severity was applied and blood was drawn. 25OHD levels were measured with Radioinmunoasay and SNP were analyzed by a standar PCR-RFLP assay.
Results: 75 asthmatics (9.6+2.5 ys) and 226 healthy children (10.2+4.8 ys), were studied. 25OHD levels were 23.9+10.7 and 22.2+7.6 ng/ml respectively (p=0.122). No significant differences were found between controlled and uncontrolled asthmatics (24.96+11.1 and 21.55+7.3 ng/ml respectively). Analysis of FokI (rs10735810) SNP revealed that all patients (16/16) requiring GINA step 4 of treatment were heterozygous for the C allele. The presence of the C allele was less frequent in patients in step 2 (30/33) and 3 (16/24) (p=0.007). No significant differences in the other SNP (rs731236) and no association of neither SNP with asthma control status, were found.
Conclusions: This pilot study established a possible association between FokI C allele and need of higher steroid doses for asthma control. VDR might play a role in asthma treatment response. Further research is required to validate it.

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The TNF-derived TIP peptide reduces lung dysfunction in experimental influenza A virus infection

Rudolf Lucas[1], Guang Yang[1], Supriya Sridhar[1], Zachary Traylor[2], Hamid Hossain[3], Bernhard Fischer[4], Trinad Chakraborty[3], Ian Davis[2]. *[1]Vascular Biology Center, Pharmacology and Toxicology, Georgia Health Sciences University, Augusta, GA, United States; [2]Veterinary Biosciences, Ohio State University, Columbus, OH, United States; [3]Medical Microbiology, Justus-Liebig University, Giessen, Hessen, Germany; [4]Research and Development, Apeptico, Vienna, Austria*

Background: Permeability edema during Influenza A virus (IAV) infection is characterized by reduced alveolar liquid clearance (ALC) and pulmonary endothelial hyperpermeability. Mortality after IAV infection is mainly due to secondary pneumococcal infections and occurs after antibiotic therapy, which can release the toxin pneumolysin (PLY) in the lungs.
Aims: To investigate whether the TNF-derived TIP peptide, which reduces PLY-induced edema, can blunt IAV-induced ALC dysfunction and combined IAV/PLY-induced barrier dysfunction.
Methods: ALC is assessed in BALB/c mice infected i.n. for 2d with 10,000 FFU/mouse (strain H1N1-A/WSN/33), using the BSA dilution method. We measure IAV/PLY-induced changes in transendothelial resistance (TER) in monolayers of human lung microvascular endothelial cells (HL-MVEC; ECIS).
Results: IAV infection reduces basal ALC by 50±5%, which is prevented by co-instillation of the TIP peptide (2.5 mg/kg; n=11). The combination of PLY (7.5 ng/ml) and UV-inactivated IAV (1 virus/cell) causes a significant drop in normalized TER in HL-MVEC monolayers from 100 to 40±3% of baseline. The TIP peptide (50 µg/ml), as well as Ro32-4032 (10 nM), a specific inhibitor of PKC-α, implicated in both ALC dysfunction and hyperpermeability, restore TER to 86±4 and 80±6% of ctrl, respectively (n=4). IAV-treatment increases PKC-α activation by 110±7% over basal in HL-MVEC, an effect significantly blunted by the TIP peptide (23±1% over basal, n=4).
Conclusions: The TIP peptide represents a therapeutic candidate for the treatment of IAV-associated lung dysfunction, since it interferes with both IAV infection-associated ALC and barrier dysfunction, upon reducing PKC-α activation.

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Oxidative stress during high altitude expedition and its influence on vessel tone-modifying mediators

Jacqueline Pichler Hefti[1], Denise Sonntag[2], Urs Hefti[3], Tobias M. Merz[4], Klaus Weinberger[2], Thomas Geiser[1], Andreas R. Huber[5]. *[1]Division of Pneumology, Inselspital Bern, University of Bern, Bern, Switzerland; [2]Metabolomics, Biocrates Life Sciences AG, Innsbruck, Austria; [3]Department of Orthopedic Surgery and Traumatology, SRO Hospital, Langenthal, Switzerland; [4]Division of Intensive Care Medicine, Inselspital Bern, University of Bern, Bern, Switzerland; [5]Center of Laboratory Medicine, Cantonal Hospital Aarau AG, Aarau, Switzerland*

Hypoxia-induced excessive pulmonary vasoconstriction is assumed to be the main cause of life-threatening high altitude pulmonary edema. Decrease of nitric oxide (NO), a potent vasodilator, has been suggested to play a significant role in hypoxia-induced vasoconstriction. To study alterations of prolonged hypobaric hypoxia, serum samples were drawn from 34 healthy mountaineers up to 6865 m during a Swiss research expedition to mount Muztagh Ata (7549 m) in Western China.
Comprehensive metabolomics analysis using a mass spectrometry-based targeted aproach revealed a pronounced systemic oxidative stress during high altitude exposure. Detecting more than 390 parameters, a significant increase of lipid peroxidation was shown. Methionine sulfoxid, determined in relation to methionine, furthermore serves as a robust indicator of oxidative stress and showed highly increased values of 30% (mean at 5500m), compared to values of 20% in septic patients. We also found relevant functional impairment of phenylalanine hydroxylase and nitric oxide synthase (NOS), enzymes which both require an oxidation-sensitive co-factor. Consequently, very low levels of NO were found. In addition, significant increase in the serum concentration of vessel tone modifiers such as leukotrienes and prostaglandins were found.
This novel and holistic approach extends the mechanistic understanding of hypoxia-related oxidative damage to a biochemical level and unravels underlying biochemical pathways involved in hypoxia-induced pulmonary vasoconstriction. Together, we demonstrate further insight into the molecular pathogenesis of hypoxia-related disorders.

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Systemic upregulation of neutrophil α-defensins and serine proteases in neutrophilic asthma

Katherine Baines[1,2], Jodie Simpson[1,2], Lisa Wood[1,2], Rodney Scott[3], Peter Gibson[1,2]. [1]*Respiratory Medicine, The University of Newcastle, Newcastle, NSW, Australia;* [2]*Respiratory and Sleep Medicine, Hunter Medical Research Institute, John Hunter Hospital, Newcastle, NSW, Australia;* [3]*Information Based Medicine, The University of Newcastle, Newcastle, NSW, Australia*

Background: The well-characterised airway inflammatory phenotypes of asthma include eosinophilic, neutrophilic, mixed eosinophilic/neutrophilic and paucigranulocytic asthma, defined by the proportion of sputum granulocytes. Systemic inflammation is now recognised as an important part of some airway diseases, but the role of systemic inflammation in the pathogenesis of asthma phenotypes remains unknown.

Methods: Induced sputum samples and peripheral blood were collected from participants with asthma (n=36). Airway inflammatory cell counts were performed on induced sputum and inflammatory phenotype assigned based on the airway eosinophil and neutrophil cutoffs of 3% and 61% respectively. Gene expression profiles were generated (Illumina Humanref-8 V3) from whole blood RNA and analysed using GeneSpring GX11.

Results: There were 6 genes classified as differentially expressed between the 4 asthma phenotypes including the α-defensins (*DEFA*) 1, 1B, 3 and 4, neutrophil proteases cathepsin G (CTSG) and elastase (ELA2). Systemic expression of *DEFA1, 1B, 3, 4, CTSG* and *ELA2* was significantly higher in the neutrophilic asthma phenotype. Microarray results were successfully validated using real-time PCR. Plasma elastase was significantly elevated in people with neutrophilic airway inflammation.

Conclusion: There is systemic upregulation of α-defensin and neutrophil protease expression in neutrophilic asthma, which may represent pro-inflammatory effects on the bone marrow and the release of immature neutrophils into the circulation. This demonstrates a systemic inflammatory component in neutrophilic asthma that further differentiates this from other asthma phenotypes, and indicates different mechanisms.

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Dysregulated miRNAs and their predicted mRNA targets in emphysematous lungs

Santiyagu Savarimuthu Francis[12], Morgan Davidson[12], Rayleen Bowman[12], Nicholas Hayward[3], Kwun Fong[12], Ian Yang[12]. [1]*Department of Thoracic Medicine, The Prince Charles Hospital, Brisbane, QLD, Australia;* [2]*School of Medicine, The University of Queensland, Brisbane, QLD, Australia;* [3]*Oncogenomics Laboratory, Queensland Institute of Medical Research, Brisbane, QLD, Australia*

There is increasing recognition of the importance of microRNAs (miRNAs) as short non-coding RNAs that post-transcriptionally regulate gene expression. Identifying the role of miRNAs in COPD would enable better understanding of disease pathogenesis and use as biomarkers for diagnostic purposes or therapeutic targets. Our aims were i) to identify miRNAs dysregulated in mild and moderate emphysema, and ii) to identify mRNAs modulated by miR-34c-5p in BEAS-2B and HFL cell lines.

Methods: i) miRNA microarray profiling (Agilent Human miRNA profiler G4470 V1.0) was performed on 29 non-tumour lung tissues obtained from The Prince Charles Hospital tissue bank. Patients were classified as mild (n=9) and moderate (n=20) emphysema according to lung function measurements (KCO and FEV_1). Technical validation was performed on the selected miRNAs using quantitative real-time PCR. ii) Genomic mRNA expression changes from transient transfection of miR-34c-5p (candidate miRNA) in BEAS-2B and HFL cells were measured using Illumina HumanHT-12 V3 arrays.

Results: COPD patients had mean (SD) age 68 (6) years, FEV_1 72 (17)% predicted and KCO 70 (10)% predicted. Five miRNAs were identified ($p<0.01$) as differentially expressed in non-tumour lung tissues in mild vs moderate emphysema patients. Upregulation of miR-34c-5p in respiratory cell lines down-regulated predicted mRNAs.

Conclusions: We have shown that miRNAs are associated with COPD severity and modulate expression of their predicted mRNAs.

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Differentiation between squamous cell carcinoma (SCC) and adenocarcinoma (AC): Expression of immunohistochemical (IHC) markers in a tissue microarray (TMA) of >1000 NSCLC

Philipp A. Schnabel[1], Esther Herpel[1], Thomas Muley[2], Hans Hoffmann[3], Peter Schirmacher[1], Arne Warth[1]. [1]*Institute of Pathology, University Clinics, Heidelberg, Germany;* [2]*Translational Research Unit, Thoraxklinik Heidelberg at the University Clinics, Heidelberg, Germany;* [3]*Thoracic Surgery, Thoraxklinik Heidelberg at the University Clinics, Heidelberg, Germany*

Introduction: Distinguishing SCC from AC has become crucial for tailored therapies of NSCLC. Many patients are inoperable at the time of diagnosis of NSCLC, >65% of the diagnoses are performed in small biopsies (Bx). The "IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma" (Travis W.D., Brambilla E. *et al.* JTO 2011; 6:244-85) firstly includes diagnostics in Bx in addition to resection specimen. We investigated the expression of IHC markers in a TMA simulating Bx.

Materials and methods: The IHC markers CK5/6, p63, desmocollin-3, CK7, TTF1, and napsin were investigated in a TMA from a primary cohort of 1005 patients with resected NSCLC.

Results: For SCC, desmocollin-3 showed the highest, CK5/6 a medium, and p63 the lowest specifity. The sensitivity of desmocollin-3 was similar to the combined sensitivity of CK5/6 and p63. Desmocollin-3 was expressed in about 85%, CK5/6 and p63 in >90% of all SCC.

For AC, TTF1 and napsin revealed a considerably higher specifity than CK7. The sensitivity of napsin did not exceed the combined sensitivity of CK7 and TTF1. CK7 was expressed in >95% of all AC, TTF1 in <90%, and Napsin in <80%.

Conclusions: To spare tumor tissue for further (e.g. molecular) analyses, diagnostic algorithms for NSCLC Bx must be established. If histology alone cannot distinguish between SCC and AC, a combination of TTF1, napsin, CK5/6 and desmocollin-3 can serve as initial diagnostic marker panel. CK7 and p63 could be used subsequently, if necessary, because of lower specifity. Currently a TMA of 300 further NSCLC is investigated, data from all 1300 NSCLC will be presented.

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DNA copy number alterations in squamous metaplastic lesions predict lung cancer

Robert van Boerdonk[1], Thomas Sutedja[2], Peter Snijders[1], Emilie Reinen[1], Saskia Wilting[1], Mark van de Wiel[3], Frederik Thunnissen[1], Sylvia Duin[1], Clarissa Kooi[2], Bauke Yistra[1], Chris Meijer[1], Gerrit Meijer[1], Katrien Grünberg[1], Johannes Daniels[2], Pieter Postmus[2], Egbert Smit[2], Daniëlle Heideman[1]. [1]*Pathology, VU University Medical Center, Amsterdam, Netherlands;* [2]*Pulmonary Diseases, VU University Medical Center, Amsterdam, Netherlands;* [3]*Epidemiology & Biostatistics, VU University Medical Center, Amsterdam, Netherlands*

No biomarker can reliably predict cancer risk in individual subjects who present with AFB-visualized premalignant lesions. Our present study was set out to identify AFB-visualized squamous metaplastic (SqM) lesions with malignant potential by DNA copy number profiling.

Within our cohort of 474 subjects at risk of lung cancer who underwent regular AFB examinations, 6 (1.3%) subjects showed rapid progression from SqM to carcinoma (*in situ*) (cases). P53, p63 and Ki-67 immunostaining patterns and arrayCGH-based DNA copy number profiles of progressive SqM lesions (n=6) were compared to those of a subset of SqM lesions of subjects who remained cancer-free (controls; n=23). Specific DNA copy number alterations (CNAs) linked to cancer risk were identified and accuracy to predict cancer in this series was determined.

While clinicopathologic characteristics and immunostaining patterns were not related to clinical outcome of SqM, the mean number of CNAs in SqM of cases (22%, range 0.48-39%) was significantly higher as compared to controls (0.09%, range 0-1.32%, $p<0.01$). Significantly more frequently altered in cases were 3p26.3-p11.1, 3q26.2-q29, 9p13.3-p13.2, and 17p13.3-p11.2 (FWER<0.10). In cases, baseline-detected CNAs persisted in subsequent biopsies taken from the initial site (median 93%, range 68-99%), and levels increased towards cancer progression (p=0.028). CNAs at 3p26.3-p11.1, 3q26.2-29, and 6p25.3-24.3 predicted endobronchial cancer risk for AFB-visualized SqM with 97% accuracy.

Our data strongly suggest that CNAs predict endobronchial cancer in individual subjects diagnosed with AFB-visualized SqM, and may be used to guide intervention to prevent lung cancer.