“Is COPD a developmental disease?”

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A developmental disease is defined as one in which normal growth and development is affected by environmental exposures acting on underlying genetic and epigenetic predispositions. COPD is a disease usually defined by irreversible obstructive spirometry, and as a term is probably no more valuable than ‘chronic renal failure’. Strictly speaking, this presentation should be entitled ‘is premature onset of airflow obstruction a developmental disease?’ to which the answer is a ringing affirmative. Indeed, recent studies have suggested a transgenerational risk, with grandparental smoking affecting grandchild risk of asthma [Chest 2005; 127: 1232-41]. The presentation is predicated on the assumption that after years of brilliant observational cohort studies, the time has come to launch an interventional study. The evidence that early childhood risk factors are important has recently been reviewed [JCOPD 2008; 5: 53-67]. A series of overlapping cohort studies have established that lung function either tracks or deteriorates, but never improves, after the preschool years [Lancet. 2007; 370: 758-64; Am J Respir Crit Care Med 2005; 172:1253-1258; N Engl J Med 2003; 349:1414-1422; Pediatr Pulmonol 1997; 23:14-20]. For normal lifelong lung function, the pre-requisites are (a) normal lung function at birth; (b) normal growth in lung function until the adult plateau at 20-25 years of age; and (c) no accelerated deterioration from the plateau. Antenatally, the factors which affect newborn lung function include maternal smoking, which causes structural effects on the developing lung [Am J Respir Crit Care 1998; 158:802-6; Am J Mol Cell Biol 2002; 26: 31-4 Am J Respir Crit Care Med 2003; 163: 140-4; Am J Mol Cell Biol 2011; 44: 222-9], and alters the fetal immune system, priming the child for a greater susceptibility to viral infections. Maternal smoking is much the most important factor. Other important influences include birth weight, maternal antibiotic and paracetamol use, and maternal diabetes and hypertension [Am J Respir Crit Care Med 2007; 175: 16-21]. There is disturbing evidence that maternal exposure to pollution also affects the fetal lung [Eur Respir J 2009; 33: 594-603]. Genes involved in antenatal lung development, such as ADAM33 and C/EBPs are important in later COPD [Am J Respir Crit Care Med 2005; 173: 55-62; Eur Respir J 2010; 35: 186-97]. Preterm birth is also an important cause of early onset airflow obstruction, and although no studies have followed survivors to late middle age, it seems likely they will have early and severe airflow obstruction. In terms of airway growth in childhood towards the adult plateau, asthma itself may interfere with this process [Am J Respir Crit Care Med 2004; 170: 234-41], also environmental tobacco smoke, traffic pollution [Am J Respir Crit Care Med 2007; 176: 377-84] and nutritional factors [Thorax 2008; 63: 234-9]. Whether viral infections cause a switch to a
phenotype of worse airway obstruction, or are a marker of previous alterations in lung structure and defence is controversial, but the evidence favours the latter. ADAM33 polymorphisms are also an important determinant of early lung function [Am J Respir Crit Care Med 2005; 172: 55]. The most important cause of accelerated decline in lung function is smoking, but again ADAM33 polymorphisms play a role [Am J Respir Crit Care Med 2005; 172: 329-332]. Adults who had episodic viral wheeze in the preschool years have an accelerated rate of decline in spirometry [Chest 2003; 124:18-24]. A study in more than 13,000 people showed that childhood disadvantage defined by any of five problems (maternal asthma, paternal asthma, childhood asthma, maternal smoking and childhood respiratory infections) predicts worse adult lung function, a faster rate of lung function decline, and a greater prevalence of COPD [Thorax 2010; 65:14-20]. Childhood factors were at least as important as adult smoking history. The Australian cohort study showed that nearly half of the children with severe persistent asthma had developed COPD by fifty years of age, again a stronger signal than smoking [Am J Respir Crit Care Med 2010; 181: A2275]. Hence one possible approach to improving lung health would be to identify a high risk group in the antenatal clinic by asking the mother three simple questions:

1. Do you have asthma?
2. Do you smoke?
3. Does the baby’s father have asthma?

and if the answer to all three is in the affirmative, then the baby is at high risk of COPD. In terms of defining an intervention, these would need to be of proven value in their own right, but would be targeted intensively.

Possible interventions could be:

- Minimising ETS exposure (cotinine measurements)
- Minimising exposure to pollution
- Monitoring weight gain in year one of life (breast-feeding)
- Preventing obesity
- Ensuring Vitamin D status is optimal

Hopefully this meeting will result in collaborations which will carry this or a similar vision forward.

“Early Life Determinants of Lung Function in Health and Disease”

Andrew Bush MD FRCP FRCPCH, Imperial College and Royal Brompton Hospital, London UK, on behalf of Janet Stocks PhD and Sam Sonnappa PhD, Institute of Child Health, London UK.

It is important to realise that infants and young children are much more vulnerable to respiratory disease than older people; and that insults to the growing lung may adversely affect lung health through life. It is essential to understand these issues, and to distinguish effects of disease from those of growth and development. Structural assessments of lung and airway dimensions do not necessarily reflect functional changes in lung growth and development, or vice versa, and despite advances in molecular, genetic and cellular research, there remains an over-riding need to investigate and interpret processes of lung growth and development at the physiological level. There are many tests of lung function, and the choice of technique is determined by the region of the
complex airway branching structure, which is being interrogated. So for example, spirometry gives information on proximal airway disease, whereas tests of gas mixing such as lung clearance index (LCI) are sensitive to distal airway problems. Changes in measured lung volume rarely reflect anatomical changes. It is not possible to distinguish alveolar number from alveoli size as the cause of lung volume changes. Measured lung volume changes may be artefactual due to airway disease. DLco may give measure of alveolar surface area but the measurement is also vulnerable to impaired gas mixing. Hyperpolarised helium is promising, but is too expensive not for big studies. The challenge is also to determine if their disease is in the silent regions of the lung (distal airways) and in the silent ages (2-6 years, now happily not so silent). We are now able to record flow volume curves in infancy (raised volume rapid thoracic compression technique), more recently only in pre-schoolers [Am J Respir Crit Care Med 2004; 169: 1152-1159.] (using incentive spirometry) and of course from school age right through into old age (conventional spirometry). The normative curves are smooth, and there are no discontinuities between techniques. LCI can be performed at all ages, and after the first year of life has the same normal range independent of age or height. This technique has been shown to be most sensitive in cross-sectional and longitudinal studies in cystic fibrosis (CF) [Thorax 2004; 59: 1068-1073; Am J Respir Crit Care Med 2005; 171: 249-56]. It does show deterioration over time in CF, and preschool LCI is strongly predictive of school age lung function abnormalities [Am J Respir Crit Care Med. 2011; 183: 752-758]. In CF, if LCI is abnormal, then structural changes on HRCT are almost invariably seen [Thorax. 2011; 66: 481-488]. In summary, we have tools which can measure lung function from birth to old age. We have a good understanding of spirometry, but it is relatively insensitive, in particular to early airway disease. LCI is more sensitive, but we lack experience in interpreting changes. However, we are well placed with suitable tools to monitor lung growth during intervention studies.

“Wnt signaling in the developing lung and during repair”

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Canonical Wnt signaling plays multiple roles in lung organogenesis and repair by regulating early progenitor cell fates: investigation has been enhanced by canonical Wnt reporter mice, TOPGAL, BATGAL and Axin2LacZ. We compared beta-galactosidase expression patterns in canonical Wnt signaling of these reporter mice in whole embryo versus isolated prenatal lungs. To determine if expression varied further during repair, we analyzed comparative pulmonary expression of beta-galactosidase after naphthalene injury. Our data show important differences between reporter mice. While TOPGAL and BATGAL lines demonstrate Wnt signaling well in early lung epithelium, BATGAL expression is markedly reduced in late embryonic and adult lungs. By contrast, Axin2LacZ expression is sustained in embryonic lung mesenchyme as well as epithelium. Three days into repair after naphthalene, BATGAL expression is induced in bronchial epithelium as well as TOPGAL expression (already strongly expressed without injury). Axin2LacZ expression is increased in bronchial epithelium of injured lungs. Interestingly, both TOPGAL and Axin2LacZ are up regulated in parabronchial smooth muscle cells during repair. In collaboration with Dr. De Langhe's group, we have shown that the activation of Wnt signaling in these cells leads to the de novo expression of Fgf10 from the
“activated” PSMC. FGF10 acts in a paracrine fashion on the cyt-P450 negative epithelial cells (also called variant of Clara cells) to trigger their amplification.

“Genetic susceptibility in COPD”

Guy Brusselle
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“Heritability of Complex Diseases and Lessons from GWAS”
Gregory Gibson
Center for Integrative Genomics, Georgia Institute of Technology, School of Biology and University of Basel, Switzerland

Heritability is the proportion of phenotypic variance in a population that can be attributed to genotypic differences. For airway diseases, estimates mainly from twin studies suggest strong genetic influences with between one and two thirds of the risk of asthma and COPD attributed to genotype, but these estimates are strongly counfounded by the methods used to control for environmental variables within populations - most notably, smoking. Both candidate gene and genome-wide association studies have been successful in identifying dozens of genetic risk factors, and these increasingly highlight the complex interplay of diverse biological pathways from immune modulation through airway remodeling to epithelial cell physiology. As with all other complex diseases, the vast majority of the genetic variance remains unexplained, most likely due to the very small attributable risks for each contributing locus. Given the primacy of infinitesimal models of the effect of common variants, a conundrum is why the incidence of asthma is either increasing or at historically high levels in so many countries. This implies genotype-by-environment interactions that themselves are likely to be too modest to detect in GWAS, yet nevertheless make a substantial contribution to risk. The potential impact of altered growth rates of components of the airway, as maternal health has improved, combined with the novel exposures in contemporary urban environments, needs to be considered, and a decanalization model for the origins of airway disease will be discussed.

“Early life determinants of asthma”

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Disordered growth during early life has been shown to result in chronic diseases such as diabetes and coronary disease in later life. Environmental factors such as unbalanced nutrition before birth result in metabolic and structural adaptations that lead to persistent modifications to offspring phenotype, i.e. fetal programming. There is evidence that respiratory disease is also influenced by fetal
programming. Asthma has been linked epidemiologically with markers of fetal growth such as anthropometric measurements at birth. Reduced fetal growth and duration of gestation are associated with impaired lung development in children. Airway function at birth is also a significant predictor of asthma, adult lung function, and possibly COPD. Genetic studies have identified a number of genes associated with asthma and also adult lung function and COPD that appear to exert their effect on lung development. Furthermore, a number of maternal environmental exposures during pregnancy e.g. tobacco smoke, micronutrient intake (Vitamin D, E) and acetaminophen (paracetamol), have been shown to increase subsequent risk of asthma in their offspring. As well as direct effects on disease risk of maternal environment during pregnancy, studies in animals and humans have shown the potential for transgenerational effects mediated by epigenetic mechanisms such as DNA methylation. In the future, increased understanding of the early life influences on lung development is needed to devise new strategies aimed at the primary prevention of lung disease in children and the optimisation of their respiratory health. This will require development of animal models to investigate biological mechanisms and test effectiveness of preventative strategies.

“Prenatal programming by maternal smoking during pregnancy may underlie susceptibility for development of asthma and COPD”

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The depressing truth is that one third of women continue to smoke during pregnancy, despite the detrimental effects of in utero smoke exposure on fetal growth and development. Maternal smoking during pregnancy is an independent risk factor for children to develop poor lung function, wheezing, physician-diagnosed asthma and respiratory infections. This also impacts development of chronic obstructive pulmonary disease (COPD), since childhood asthma, poor lung function and respiratory infections in early life all are risk factors for COPD. All these data indicate that the origins of asthma and COPD may be initiated by critical in utero events. Similarly, a transgenerational effect of smoking during pregnancy on the development of asthma may occur, since grandmaternal smoking during pregnancy was associated with an increased asthma risk in her grandchildren. This effect is consistent with a heritable, epigenetic process, such as DNA methylation.

Recently, the group of Hylkema et al. (Groningen, The Netherlands) found that newborn (Day 1) mice from mothers exposed to cigarette smoke during pregnancy had a lower expression of genes, belonging or related to the Wnt signaling pathway, a pathway important in lung development. They also observed more airway remodeling in adult non-smoking offspring from these smoking mothers, i.e. increased smooth muscle mass and collagen deposition, a finding typical for asthma and COPD. Altered expression of genes can result from altered DNA methylation induced during in utero smoke exposure. Preliminary data by the group of L. Kobzik (Boston, US), analyzing human fetal lung samples from smoking compared to non-smoking mothers, indeed showed altered methylation in the promoter region of certain genes which are relevant to airway remodelling. These data indicate that early life effects, such as maternal smoking, may permanently alter the expression of fetal genes through epigenetic modifications, setting the stage for higher susceptibility to develop asthma and COPD.
“Maternal genetic asthma predisposition affects pulmonary microRNA profiles in neonatal offspring”

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Exposure induced deregulation of microRNAs (miRs) during early critical developmental periods has been proposed to contribute to the propagation of asthma risk in later life. Therefore, we asked if maternal genetic asthma predisposition is sufficient to affect pulmonary miR profiles in offspring that do not bear the genetic asthma risk. To address this question, we used female mice with a heterozygous deficiency for Tbx21 as they develop spontaneous airway remodeling and airway hyperreactivity (Finotto et al., Science, 2002; 295:336) and mated them with WT males. Neonatal lungs from male WT offspring of dams with and without genetic asthma predisposition were removed within 24h after birth and total mRNA including small RNAs was extracted. Duplicate pools of RNAs were subjected to miR expression profiling (ABI, TaqMan® Array Rodent microRNA cards). In silico target prediction was performed for miRs with a >1.5 fold change followed by pathway analysis (DIANA-mirpath, TargetScan). Male WT offspring of asthma-prone dams showed an up-regulation of 13 of 641 miRs (1.5-2.1x), while 17 miRs were down-regulated (1.5-4.7x) compared to male WT offspring of dams without genetic asthma predisposition. Pathway analysis showed a significant enrichment of target genes within the WNT pathway (49 of 154 genes). MiRs 27a* and 124 were found to target multiple genes (≥15) in the WNT pathway suggesting a key regulatory function for these miRs in WNT signaling. These data indicate that maternal genetic asthma predisposition affects miR profiles during an early developmental stage and might therefore influence lung development.

“WNT signaling in COPD and interstitial pulmonary fibrosis”

Melanie Königshoff

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“Developmental pathways in asthmatic airway remodelling”

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Airway remodelling is a feature of chronic asthma, characterised by subepithelial fibrosis; increased myocyte smooth muscle mass; and mucous gland hyperplasia. Although airway remodelling has been associated with chronic asthma it remains unclear whether it develops as a result of chronic cycles of inflammation in response to allergen exposure or in parallel with allergic lung inflammation. In
support of this latter hypothesis is the finding that biopsies taken from even very young children with preschool wheeze show evidence of lung remodelling. In addition, treating airway inflammation does not always reduce or prevent development of remodelling. We have developed a model of neonatal allergic airway disease in order to investigate the molecular mechanisms underlying early development of airway remodelling and lung dysfunction. This model enables us to assess changes in structure and function in the context of normal post natal lung growth & development as well as the maturing immune system. Neonatal mice exposed to allergen soon after birth show AHR and remodelling occurring with Th2 type inflammation, and unlike in a similar adult model of disease, the remodelling and inflammation develop in parallel, rather than sequentially. We have determined that the route of allergen exposure is critical when assessing the contribution of mediators such as TGF-β. Blockade of TGF-β in a traditional peritoneal sensitisation lung ovalbumin exposure model results in abrogation of airway remodelling. In contrast, neutralisation of TGF-β during an inhaled house dust mite challenge protocol has no effect on airway remodelling but exacerbates airway hyperreactivity and inflammation. Our data underscore the importance of developing mouse models that are relevant to clinical disease phenotypes, and suggest that age is a confounding factor in the underlying disease mechanism. In future, disease models need to reflect not only genetic influences, but also environmental influences such as age, diet, infection history and allergen exposure when investigating interactions between resident pulmonary cells and infiltrating immune cells.

“Hedgehog signaling pathway in lung fibrosis”

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Idiopathic pulmonary fibrosis (IPF) is a devastating disease of unknown etiology associated with the accumulation of extracellular matrix (ECM) and fibroblasts in the distal airways. It is recognized that no satisfactory treatment currently exists. Identification of new therapeutic targets in IPF is required. The Sonic Hedgehog (SHH) signaling pathway is strongly involved in epithelial cells-fibroblasts interaction during fetal lung development. In the SHH canonical pathway, the binding of SHH to its receptor Patched1 (PTC) relieved the PTC-mediated inhibition of the protein transducer Smoothened (SMO) in the primary cilium, allowing SMO to promote GLI-dependent transcription. We hypothesized that the Hedgehog (HH) pathway played a key role in epithelium-fibroblast interactions during the alveolar repair process and during lung fibrogenesis in the adult lung. The expression of key members of the HH pathway is detected in lung biopsies from control and IPF subjects. We showed that the HH pathway is reactivated in IPF lung tissue ex vivo. In vitro, addition of direct agonists of the HH pathway had no major effect on primary lung fibroblasts (HLF). Importantly, we deciphered the crosstalk between the HH and TGF-β pathways in HLF. TGF-β modulated the expression level of key components of the HH pathway in HLF. We showed that SMO was required for the TGF-β induced myofibroblastic differentiation of control HLF while IPF lung fibroblasts were partially resistant to SMO direct inhibition. Meanwhile, our results
demonstrated that activation of the canonical GLI pathway in the primary cilium as well as GLI-dependent transcription in the nucleus were required to induce myofibroblastic differentiation of both control and IPF HLF stimulated with TGF-β1.

These data support a pro-fibrotic action of the HH system in IPF and identify the GLI transcription factors as potential therapeutic target in IPF.

“Early immune responses to the environment”
Benjamin Marsland

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“Can we explain sex differences in chronic lung disease?”
Dirkje Postma, Prof of Pulmonology, University Medical Center Groningen, Groningen Research Institute for asthma and COPD (GRIAC); NL

Asthma and COPD are both respiratory diseases with increasing prevalence worldwide. Of interest is the notion that not only the prevalence, but also the severity and mortality differs between males and females with these diseases. Thus severe asthma predominantly occurs in females and early onset COPD (reflecting also severe disease) is occurring more in females than males. Autoimmune diseases are more prevalent in females than males and it is an unsolved question whether differential immunologic processes are the origins of the gender differences.

Both genetic and environmental factors may contribute to asthma and COPD and environmental exposures may be different between genders, since it is known that boys play more outside than girls, which will affect their exposures. Some genes have been associated with asthma in females only, whereas others are associated with disease in males preferentially.

The underpinnings of gender differences may already stem from in utero lung development, where this is significantly different in timing of maturation, branching morphogenesis, and production of e.g. surfactants which may improve lung unfolding after birth. This is then continued after birth with differential lung growth and halting of growth in males and females. Of interest are the small airways in the lung in this respect; they are the largest part of our lung breathing area, and these small airways thus contribute to airway obstruction very significantly. It has become clear that small airway disease is present in both asthma and COPD, However, in how far gender differences exist in this respect is yet unsolved. Today there is an unmet need to better diagnose, assess severity of small airway disease and particularly investigate whether these small airways can be targeted with small particle size treatment and if this then would benefit patients in current control of their disease and future outcome. It is needed to better assess gender differences in origins of lung development, involvement of the small airways and their interrelationship. To this aim we need collaboration.
between clinicians for childhood and adult respiratory care, biologists, immunologists, geneticists, studies in animal models and in vitro systems.

“Determinants of fetal and infant growth on respiratory symptoms”

Professor Graham Roberts, Division of Developmental Origins of Health and Disease, Faculty of Medicine and Health and Life Sciences; University of Southampton School of Medicine, UK.

Little is known about whether patterns of early growth are associated with altered respiratory and immune development. Our data relates prenatal and infant growth patterns to wheeze and atopy at age 3 years. Birth weight and length were measured in 1548 children born at term. Conditional fetal head and abdominal circumference growth velocities were calculated from antenatal ultrasound measurements. Conditional postnatal growth velocities were calculated from infant weight, length and adiposity data. Measures of size and conditional growth were related to parentally reported infant and early childhood wheeze and to atopic status at age 3 years.

The risk of atopy increased by 46% per SD increase in abdominal circumference growth velocity from 11 to 19 weeks gestation but by 20% per SD decrease in abdominal growth velocity from 19 to 34 weeks (p=0.007 and p=0.011, respectively). The risk of atopic wheeze increased by 20% per SD decrease in 19 to 34-week abdominal growth (p=0.046). The risk of non-atopic wheeze increased by 10% per SD decrease in 11 to 19-week head circumference growth. Greater relative infant weight and adiposity gains were associated with both atopic and non-atopic wheeze.

A rapid growth trajectory during 11 to 19 weeks gestation followed by late gestation growth faltering is associated with atopy, suggesting that influences affecting fetal growth may also alter immune development. A lower early fetal growth trajectory is associated with non-atopic wheeze, possibly reflecting an association with smaller airways. An association between postnatal adiposity gain and wheeze may partly reflect prenatal influences that cause fetal growth to falter but are then followed by postnatal adiposity gain.

Pike K Thorax 2010;65:1099e1106. doi:10.1136/thx.2010.134742

“Mechanisms of epithelial morphogenesis: A genome-wide analysis of airway maturation in Drosophila”

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The respiratory tubes of mammalian lungs and the Drosophila tracheal system undergo a series of maturation events at the end of embryogenesis. During this period the nascent tubes acquire their mature size, clear the luminal liquid and transform into functional respiratory networks. We have recorded three precisely controlled transitions of cellular activities during airway maturation in living fly embryos: First, a secretion burst deposits extracellular matrix into the lumen and expands tube diameter. Second, the activation of a massive apical endocytosis wave clears the matrix. Finally, luminal liquid is evacuated and the network is filled with a gas within ten minutes. We have
characterized several genes required for each maturation step, but the mechanisms underlying the precise spatial and temporal regulation of epithelial activities during airway maturation are unknown.

To address the regulation of airway maturation we used a tracheal specific driver and 18036 transgenic UAS-RNAi strains to first identify all protein-coding genes involved in the process. We screened for defects in larval tube maturation to maximize RNAi knockdown efficiency and exclusively target zygotic gene expression. We identified 1461 genes, (13.4%) involved in tube maturation.

Tracheal inactivation of 1935 of the remaining genes caused lethality or adult phenotypes in >50% of the animals. We used GOstat to find overrepresented classes of biological gene ontologies among the 1461 genes in the tube maturation group. 980 of the positive genes could be assigned biological gene ontologies. Genes involved in transport of proteins or ions, epithelial junction assembly, cytoskeletal organization, metabolic processes and RNP biogenesis are enriched in this set. We have confirmed the RNAi knockdown phenotypes of 1072 selected genes by examining independent UAS-RNAi strains.

We are now extending the phenotypic classification of the positives into functional groups ("secretion", “protein clearance”, “gas-filling”) using the integrated UAS-ANF::GFP marker.

Our main interest is to identify the developmental regulators of airway maturation. We classified 1172 “tube maturation” genes according to the molecular function annotation of the predicted proteins and the potential presence of *H.sapiens* and lack of *C.elegans* orthologs. This analysis identified 64 genes encoding proteins of unknown function (48), transcription factors (10), kinases (4), GPCR (1) and channels (1). We hypothesize that this set of genes may represent phylogenetically conserved regulators of the cellular events of airway maturation.

**“MicroRNA body map: dissecting miRNA function through integrative genomics”**
Jo Vandesompele

Center for Medical Genetics, Gent University Hospital, Belgium

See: The microRNA body map: dissecting microRNA function through integrative genomics.

**“Why classical genetics does not give the right answer”**
Matthias Wjst

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The world has seen a massive increase of genome-wide disease association studies, probably >1000 since the first publication in 2005. Many authors, however, are already trying to deflate the genomic bubble as genetic risks are usually small and replication studies overlap only by a minor fraction. I am highlighting some asthma studies where lead association signals do not explain linkage, genomic regions are being poorly defined while it has been impossible to find any functional variant.

With only a few skeptical voices in 2005, the choir in 2011 is now large, chanting about the “missing heritability”. Unfortunately, even that may be a misnomer as it is more about “unexplained heritability”. Some authors concluded, however, that the heritability might have been overestimated,
some believe in the long tail of common variants, others in missed rare variants, epistasis, epigenetic effects or simple phenotype heterogeneity.

I am highlighting a roadmap to overcome these challenges that will probably require another decade of enormous efforts to decipher the genetics of complex human diseases. Refining phenotype definitions may be more relevant than only increasing sample sizes. Laboratory approaches include deep sequencing of whole genomes, bisulfit sequencing of various tissues at repeated time points, RNA sequencing from target tissues and novel statistical approaches combining data including GxG, GxE and ExE interaction models.

There are infinite variations in human phenotypes and while humans look different from the outside, it is likely that they carry as much genetic and epigenetic variations inside.