Optimizing Experimental Research in Respiratory Diseases: an ERS Statement

The online supplement provides complementary and detailed information in the following domains of the Task Force. These supplements are based on the earlier working documents to generate the main report. The Task Force members felt strongly that the information shown here could be highly valuable as reference documents to the research community. Some areas that were covered in the main report are not covered in the supplement, because the working documents did have a different draft format and were not suitable for publication.

1) Anesthesia and euthanization of rodents
2) Pre-analytical conditions of tissues/cell harvesting/ collection/storage
3) Large animal models for respiratory diseases
4) Chronic Obstructive pulmonary disease (COPD) including clinically relevant subgroup of COPD, environmental factors and exacerbations
5) Infection/Pneumonia

1) Anesthesia and euthanization of rodents

The most frequently employed short-term injection anesthesia which can be applied via intravenous, intramuscular, or intra-abdominal routes to mice and rats as well as dogs, cats, sheep, and horses is a combined ketamine hydrochloride and xylazine hydrochloride anesthesia, which may be combined with the dopamine 2 receptor blocker azepromazine (Jaber et al.). Although significant species-specific differences in dosage regimens exist, for anesthesia in mice, ketamine should be applied in a concentration range of 50-100 mg/kg body weight (b.w.), while xylazine hydrochloride should be used at final concentrations of 2.5-8 mg/kg of b.w., as both drugs dose-dependently cause bradycardia and bradypnea, which can be reversed by yohimbine [1-4]. In case re-dosing of anesthesia is required, mice should receive either 50 % of the initial ketamine dose, or 25 % of the initial combined ketamine-xylazine dose as soon as the pedal withdrawal reflex starts to return [5].
Commonly employed volatile anesthetics in laboratory animals include isoflurane (Baxter, Unterschleissheim, Germany), sevoflurane, and desflurane (Suprane, Baxter) [6]. The advantage of

inhalational over injection anesthesia is its simple titratability of anesthetic depth and its very rapid recovery, which qualifies it for use for long-term anesthesia, or for repetitive anesthesia within short time periods [7].

Beside its role as inhalational anesthetic, isoflurane is frequently used for the sacrifice of mice and rats [8], which according to recommendations of the Federal European Laboratory Animal Science Association (FELASA), must be accompanied by immediate blood withdrawal from the Vena cava or cardiac puncture to ensure death of the laboratory animal. Alternatively, due to low costs and ease of availability, many researchers still make use of carbon dioxide (CO2) to euthanize laboratory animals. However, compared to volatile anesthetics such as isoflurane, use of CO2 for euthanasia has several disadvantages, as inappropriate use of CO2 may lead to major hypercapnic excitation of animals at the end of the experiment. Second, exposure to CO2 leads to a reduction in pH in the airways and peripheral blood of both rats and mice, which as a consequence adversely affects the pharmacokinetics of e.g., basic drugs, which is not observed when isoflurane is used for sacrifice [9]. A most recent report also demonstrates adverse effects of CO2 euthanasia of mice for the subsequent generation of mouse embryos using in vitro fertilization approaches [10]. These data imply that wherever possible, available alternatives to CO2 euthanization of laboratory animals should be used. As another alternative method to euthanize mice, cervical dislocation (CD) is frequently employed, although more recent studies raised concerns as of its efficacy as successful method for euthanization of mice [11]. Since CD requires specific training, it is recommended that CD should only be performed by well-trained lab personnel to ensure its correct execution.

2) Pre-analytical conditions of tissues/cell harvesting/ collection/storage

It is important not to overfix tissue/cytological preparation if immunohistochemistry (revelation of antigen by specific antibody) is to performed. For histological techniques, the choice of the fixative and duration of fixation need to be optimised [12, 13]. While in research a combination of 4% formaldehyde with 0.1% glutaraldehyde in 0.2 M HEPES buffer followed by freeze substitution in 0.5% uranyl acetate in methanol have been recommended [14], other fixatives (such the ones used in the clinical setting) namely buffered 4% formaldehyde alone is also applicable and fixation should be kept short (12-24 hours for small fragments, 48 hours max for larger fragments) and uniformed within the groups studied.

Similarly, cytology preparations either form BAL cytopsins or cell cultures should be fixed either in 10% acetone for 10 minutes, 4% buffered formalin for 5 min, or PBS- Ethyl alcohol for 30 seconds, depending on what techniques are used subsequently [15].

Cytology slides can be kept at +4 degrees (fridge) for a few days or at -20 degrees for a few months.

Antigenicity (expression of antigens at the surface of the cells) does fade overtime, and on old stored slides, testing for preserved/retained antigenicity might be needed before applying immunohistochemistry.

Animal lung tissue after fixation (12-24h max) should undergo "processing" readily as it is best practice to keep paraffin embedded tissue blocks and only cut slides upon the need for immunohistochemistry rather than storing cut unstained slides. Tissues are processed in a vacuum infiltration processor (VIP) automated tissue processor. This is based on tissue being exposed to increasing concentrations of ethanol (50% to 100% over a few hours), then in xylene/toluene and finally in liquid paraffin at 60 degrees. Researchers should be encouraged to liaise with their local hospital histopathology department to streamline this process.

One dehydrated and cleared, the tissue is embedded in melted paraffin, that once hardened, provide a hard support for tissue slides sectioning on glass slides, usually at 4-5um thin.

If immunohistochemistry (IHC) is being performed, slides should be cut on positively charged (sialinised) slides to prevent detachment of tissue section from the slide during antigen retrieval and immunohistochemical protocol. These sialinised slides are either commercially available but glass slides can also be sialinised using a solution of 3-aminopropyltriethoxysilane (APES).

Cut slides should be put in an oven at 56 degrees for 20 min and then used for immunohistochemistry of short-term storage in a dry cool dark area for a few months.

Resources such as IHC world [16] are available on line to facilitate and optimise tissue processing. Studies have demonstrated that the profiles of these molecules can change drastically during transport and storage thus making a reliable diagnostic or pharmaceutical research unreliable or even impossible.

Similarly, optimising pre-analytical steps will provide good quality RNA, DNA and protein for molecular studies. International consortiums such as SPIDA are promoting standardisation of pre-analytic conditions, quality assurance schemes and innovative pre-analytical tools by establishing guidelines in sample collection, handling, stabilisation, purification and storage of clinical samples that should be applied to research samples [17] Reducing or eliminating pre-analytical errors that

lead to inaccurate results can be achieved by integrating and standardizing workflows for the collection and processing of samples to increase analytical accuracy and laboratory efficiency and developing automated sample preparation when feasible.

3) Large animal models for respiratory diseases

3.1 Chances and limitations of large animal models in respiratory research

Despite 17 Nobel prizes were awarded to scientists that studied cattle, horses, sheep or poultry as models for biomedical research [18], the vast majority (about 98-99%) of animal experiments are currently undertaken with rodents, predominantly mice. Interestingly, animal models based on domestic animals or livestock have gained increasing attractiveness during the last decade, and are currently re-introduced as an essential part of biomedical research. Table 6 (in the main document) summarizes large animal models for non-infectious and infectious respiratory diseases.

In principal, there are three kinds of animal models suitable for biomedical research: natural models, experimentally induced models, and transgene models:

- *Natural models* are based on naturally occurring pulmonary diseases with similar pathophysiology in animals and humans. Typical examples are feline asthma, "ski asthma" in sled dogs, RSV infection in calves, bovine tuberculosis, or naturally acquired *Chlamydia* infections. Species-specific peculiarities in the pulmonary vasculature pre-dispose calves as a model for pulmonary.

- *Experimental models* in pulmonary research can either simulate the natural diseases under defined conditions or have been introduced for distinct purposes, for example ventilated models of MRSA-induced pneumonia in sheep or swine.
- With steadily increasing possibilities to generate genetically modified large animals, even *transgene models* become available in addition to mice models. The pig model of cystic fibrosis is one example [19-21]. Superior to the mouse model, the pigs lacking CFTR exhibit defective chloride transport and develop typical signs of CF seen in humans. In addition of serving as *in vivo* models of complex pathophysiological functions, genetically modified animals will be a

future source of primary cells for *ex vivo* examination of biological functions at a cellular level [22].

Large animal models are more expensive, laborious and time-consuming, and the experimental use of pet and livestock animals receives lower ethical acceptance compared to laboratory animals. Nevertheless, the choice of an animal species to be used as a model should be based primarily on the biological relevance according to the current state of knowledge instead of convenience and reduced cost.

Large animal models offer unique changes that will complement existing models in rodents while taking into account the 3R principles (*refinement*, *reduction*, *replacement*) as introduced by Russell & Burch more than 50 years ago [23]:

- *Chronicity & long-term studies.*

  Because of their limited life span, rodents are not suitable to study the complex pathogenesis of chronic diseases with a manifestation that may last for years. In contrast, a considerably long life-span of large animals supports investigations on chronic diseases and on chronic infections. Typical examples are naturally occurring chronic obstructive pulmonary diseases in animals (asthma in cats, chronic obstructive pulmonary diseases in horses) partially share pathogenetic features with human diseases. Also, chronic infections with so-called ‚atypicals' (e. g. *Chlamydia* spp., *Mycoplasma* spp.) in bovines present suitable animal models in natural pathogen-host settings. The latter provide strong evidence that persistent and recurrent chlamydial infections are associated with chronic airway obstructions [24].
- *Complexity & system biology.*

  Larger animals offer the great potential to perform long-term functional studies allowing a simultaneous within-subject approach of functional, inflammatory and morphological changes. Different samples (for example, blood, BALF, tissue biopsies, exhaled breath) or biological variables can be perfectly analysed in their interactions if derived from one subject over time. This is particularly valuable when following system biology approaches.
- *Biological variability of data sets & sample size.*

  Parallel assessments of multiple parameters in intra-individual follow-ups over time minimises the number of experimental animals as well as data variability. In a biological setting, intra-individual variation accounts for only 25 – 30 % of the total variability of

physiological parameters. By contrast, variability between subjects is generally much higher (by 3 – 4fold). In consequence, data obtained in intra-individual kinetics are less variable compared to inter-individual baseline data, and the number of animals required for a meaningful statistical analysis can be kept much lower than in group comparisons.

- *Biological relevance.*

  Significant differences exist between species with respect to the genetically determined regulation of defence mechanisms. For example, interleukin-8 (IL 8) plays a significant role in inflammatory processes. However, the il-8 gene is missing in the mouse genome. It does exist in the genome of dogs, pigs, sheep, and cattle. The protein encoded for IL 8 even exhibits a high cross reactivity between those species [25, 26]. Intriguingly, the bovine genome, fully sequenced in 2009, more closely resembles the human genome than it resembles that of mice and rats. Approximately 80 % of the genes identified in the bovine also exist in other mammals [27]. Beyond the genetic background, structural and physiological similarities of the porcine and human airways, e.g. with regards to the local immune system [28, 29] or the composition of the epithelial surface liquid [30] qualify pigs as suitable models for human airway diseases. Pigs can be used to study gene transfer events in the lung in the development of gene therapies for lung diseases as cystic fibrosis [31]. Species lacking collateral airways, and presenting a strong segmental anatomy of the lung (e. g. pigs, cattle) are particularly suited to mirror pulmonary dysfunctions associated with airway obstructions [32].

- The development of immunological competence during fetal ontogenesis and in the neonate (immuno-physiology) is similar in larger animals species compared to humans. Thus, livestock models are predestined to study maternal-fetal interactions and immunological mechanisms during post-natal development [26]. In this context, the asthma model in dogs is highly relevant to elucidate maternal influences in the transmission of asthma susceptibility [33].

3.2 Modes of experimental challenges

Different modes of exposure are available with each presenting significant advantages and disadvantages. The most frequently used are given in Table S1 with typical examples of application. As exemplarily shown for lung infections with *Chlamydia*, large animal models of respiratory

infections strongly support a deeper understanding of the host-pathogen interactions in a complex pathogenesis, and allow evaluation of clinically relevant treatment options [34-36].

3.3 Read out parameters *in vivo*

Blood gas analysis and assessment of acid base status

To assess the efficacy of pulmonary gas exchange, arterial blood gas analysis is still the gold standard. Practically, access to arterial blood depends on the animal species. In horses, arterial blood can be easily collected from *A. carotis* while this procedure is difficult or even requires surgery in ruminants and pigs.

Collecting blood samples in conscious animals by puncture may induce stress or pain, and consequently hyperventilation. The latter, however, could reduce the actual degree of hypoxaemia or even could make hypercapnia invisible. Thus, catheterization of an arterial blood vessel is highly recommended to facilitate repeated sampling of arterial blood in ongoing studies by minimizing stress for the animals. As an example, catheterization of *Aorta abdominalis* has been successfully implemented in respiratory infection studies in calves [37, 38]. Any reduction of stress while collecting blood from the animals increases the quality of the samples.

When analyzing blood gases or blood pH, one has to be taken into account that analyzers usually work at 37°C which is the normal body temperature of human beings. Physiologically, all animal species present significantly higher body temperatures, sometimes above 39°C. A difference of about one degree in body temperature, however, corresponds with a change of about 0.5 kPa in partial pressures of blood gases. Therefore, blood gases and pH always need to be corrected for the actual body temperature measured immediately before or after blood collection.

If there is no access to arterial blood, some valuable variables of acid base status can be obtained from venous blood as described for pigs calves experimentally infected with viruses or bacteria [39, 40].

Pulmonary function testing

Using a tightly fitting face masks of the appropriate size, all techniques of pulmonary function testing (PFT) known from human medicine are principally available to be used in an awake, spontaneously breathing large animal with the exception of active breathing manoeuvres. Spirometry can be used to assess variables of spontaneous ventilation (i.e., tidal volume Vt,

respiratory rate f_R, minute ventilation Vmin). Combined with capnography, i.e. CO_2-exhalation versus exhaled volume, dead space volume (Vd), and the ration Vd/Vt can be assessed, and alveolar ventilation can be calculated. Re-breathing methods based on multi-breath analyses are helpful to measure functional residual capacity (FRC) by using Helium-wash-in or –wash-out, or assessing the diffusion capacity based on CO-transfer [41] [38].

To evaluate respiratory mechanics in large animals, three principal techniques have widely been used in many species of domestic animals. (i) The classical approach calculates pulmonary resistance (RL) and dynamic compliance (Cdyn) from measuring oesophageal pressure via oesophageal balloon while flow and volume derive from pneumotachography. (ii) Forced oscillation techniques bear the advantage of being non-invasive and are capable of distinguishing between obstructive and restrictive ventilation alterations but at the same time differentiates between obstructions of the proximal and peripheral airways by measuring complex respiratory impedance (Zrs) or its components, i.e. respiratory resistance (Rrs) and respiratory reactance (Xrs) in a frequency range appropriate to the lung size of the animal: Reports from experimental models of lung infections are exemplarily available for pigs [42] and calves [38, 40]. (iii) Bodyplethysmography has been described for piglets [43], sheep [44], cats [45], and dogs [46]. Comparable to the use of this technique in mice or rats, the outcome parameter is ‚Penh'. This parameter provides rather non-specific information associated with a changing breathing pattern, but does not allow differentiating restrictive from obstructive ventilatory disorders.

Technical solutions for measuring pulmonary function data strongly depend on the size of the animal. Taking this aspect into account, equipment of human pneumology is often directly applicable to animals weighting about 20 to 100 kg, i.e. calves, sheep, goats and pigs to assess pulmonary functions non-invasively in conscious animals while breathing spontaneously. Measuring identical parameters as in humans, direct comparisons between data obtained in large animal models and data obtained in patients become possible. Due to their non-invasive character, they are of beneficial value with regards to animal welfare protection.

Biological specimens from the respiratory tract

The diagnostic repertoire obtaining biological samples from the lungs of large animals is widely identical to human medicine, which also allows direct comparisons and translation of results. Bronchoscopy can be applied to larger animals while being conscious and in standing position

(horses, cattle), but requires anaesthesia in pigs, cats or dogs. Focussing on translational respiratory medicine, anatomy and bronchoscopy of the porcine lung have been described [47]. Bronchoscopic sampling techniques include broncho-alveolar lavage, epithelial brushing, and tissue biopsies as recently described and illustrated for the bovine lung in a video publication [48]. Due to the size of large animals, all these biological samples may be taken repeatably in sufficient amounts without having to sacrifice the animal.

Exhaled breath and exhaled breath condensate

Collection of exhaled breath (EB) and/or exhaled breath condensate (EBC) for further analysis is a current research option directly comparable to its use in human medicine when searching for exhaled biomarkers. Analysis of volatile organic compounds (VOC) has been implemented in large animal studies to identify respiratory infections. Methods of VOC analysis applied to large animals include (i) GC-MS to identify biochemical compounds [49], and (ii) methods of pattern recognition using DMS/IMS [50] or electronic noses. The high level of standardization in large animal studies that can never reached in humans with respect to ambient conditions, nutrition etc. qualifies these models to elucidate physiological background and physiological variability of VOC candidates that are of interest for future diagnostic purposes [51, 52]. With respect to EBC, studies focussing on collection and analysis in domestic animals contributed significantly to understand the strengths and methodological limitations of this diagnostic technique in a general sense – independent of any species [53, 54].

Imaging techniques

Although ultrasound imaging of lungs is possible in many large animal species, it has been most frequently applied to pigs and calves. Using pigs as models, lung sonography has been recommended as a very accurate method in diagnosing even small amounts of intrapleural air in cases of pneumothoraces [55], or - combined with lung flooding – for successful tumour detection [56]. Furthermore, the pig has been exploited as a useful model for ultrasound-guiding endoscopic lung surgery or to evaluate high-intensity focused ultrasound as a potential new strategy for treating lung cancer [57]. In calves in experimentally induced lung infections, the diagnostic value of sonography to identify pneumonic lesions was characterized in comparison to pulmonary function

testing and clinical examination [58]. As in clinical pneumology, X-ray, scintigraphy, and computed tomography [44] are further techniques applicable to large animals.

3.4 Conclusions

Large animal models form an innovative part of modern interdisciplinary biomedical research. For use in translational medicine and as comparative models, these models offer the distinctive opportunity to obtain results with high biological relevance and, in various instances, even of dual use (to improve human as well as animal health). Although in good agreement with the 3R concept and the requirements of animal welfare, models deploying domestic animals suffer from low ethical acceptance. In contrast to rodent models, large animal models are more demanding regarding time and financial resources. However, biologically relevant livestock species models – with the animal species investigated being selected according to the particular requirements of the scientific questions to be solved - embedded into the communication and collaboration between several disciplines from human and veterinary medicine inhere the outmost potential to make progress according to the ‚One Health' concept.

Table S1: Modes of challenges applied in large animal models

Application mode	**Pathogen**	**Animal species used as model**	**Advantages**	**Disadvantages**	**References**
nasal instillation (drop or aerosol)	Virus (hRSV)	non-human primates	easy to perform	exposure of only extra-thoracic upper airways	[59]
intratracheal (bolus)	*Pasteurella* LPS	calf non-human primates	defined doses	spontaneous coughing is often induced; undefined distribution of the inoculum	[60]
intrabronchial inoculation (visually guided)	*Chlamydia*	calf	defined doses at defined locations	anaesthesia of the animal required, technically challenging (videobronchoscope and trained personnel required), time consuming	[48, 61]
aerosol (inhalation)	Virus (bRSV) *Mycoplasma* *Chlamydia*	calf calf pig	non-invasive, spontaneous breathing, exposure of upper and peripheral airways	unknown quantity of deposition	[62] [63]
parenteral a. intramuscularly b. intravenously	a. Virus (PRRSV) b. LPS	a. pig b. pig, calf		suitable for (a) pathogens with high affinity to the respiratory tract only, or (b) for systemic challenges	[64, 65]

4) CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

4.1 How do we define COPD for experimental research (what do we want to model)?

According to the Global Initiative for Chronic Obstructive Lung Disease, "*COPD is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced inflammatory response in the airways and lung to noxious particles and/or gases"* [66]. In an individual patient, varying degrees of chronic bronchitis, bronchiolitis, and emphysema contribute to chronic airflow limitation [67-69]. Exacerbations and comorbidities contribute to the overall severity of the disease[1].

In recent years, findings obtained in large cohort studies have provided longitudinal insight into the evolution of airway obstructions and its role in the development of COPD. An accelerated rate of decline in forced expiratory volume in 1 second (FEV_1) has long been regarded as a key feature of COPD [70]. In the ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints) study, the rate of change in FEV_1 over 3 years was found to be highly variable, with only 38% of patients showing an elevated rate of decline of FEV_1 of more than 40 mL/ year. In close to a third of all patients, there was no decline in lung function over the study period [71]. Moreover, in a combined analysis of 3 cohort studies, it was found that approximately half of the patients with COPD had already a low FEV_1 in early adulthood, with a normal decline in FEV_1 afterward, despite similar smoking exposure [72]. This study emphasizes the importance of lung growth and the early origins of COPD [73]. Last but not least, it has become increasingly clear that current and former smokers with preserved lung function may still present with respiratory symptoms, exacerbations, activity limitation, and evidence of airway disease [74].

Attention has been paid to tobacco smoking as a major cause of COPD. Accordingly, the inflammatory response to tobacco smoke has been well characterized, with involvement of the innate and adaptive immunity [75-77]. It is widely accepted that COPD results from a gene-environment interaction [78]; among people with the same smoking history, not all will develop COPD. The best-documented genetic risk factor to date is alpha-1 antitrypsin deficiency [79, 80]. Several genome-wide association studies have discovered genes that are associated with both the presence of the disorder and the severity of airflow obstruction [78]. In recent years, it has become evident that, in major parts of the world, COPD can be caused by exposure to biomass fuels [81]. Other risk factors that have been implicated in the pathogenesis of COPD are occupational exposure to dust and gases, respiratory tract

infections during childhood, outdoor air pollution and poor socioeconomic status, as well as a history of pulmonary tuberculosis and chronic asthma [73].

The natural course of COPD is punctuated by periods of disease exacerbation [66, 82, 83]. Clinically, exacerbations are defined as "*a sustained worsening of symptoms from the patient's stable COPD state, beyond normal day-to-day variations, which necessitates a change in medications*" [84]. Based on the ECLIPSE-study, it appears that some patients present with frequent exacerbations (i.e. $\geq$ 2 exacerbations per year) and that this frequent exacerbation phenotype is relatively stable over a 3 year period [82]. Four distinct biological clusters have been associated with COPD exacerbations, including bacteria-predominant, eosinophil-predominant, viral-predominant, and pauci-inflammatory [85].

Comorbidities in patients with COPD have an important impact on disease severity and patient survival [66, 86]. Different clusters of clinically important and objectively diagnosed comorbidities have been reported. Cluster analysis revealed five distinct clusters: a cachectic cluster, a metabolic cluster, a cardiovascular cluster, a psychologic cluster and a subgroup of patients with significantly less comorbidity [86]. Systemic inflammation was found to be present in a limited proportion of patients, e.g. 16% of patients with COPD in the ECLIPSE study [87]. The presence of elevated levels of CRP, fibrinogen and leukocyte counts in individuals with COPD is associated with an increased risk of having exacerbations [88].

Taken together, COPD is a complex condition, encompassing a spectrum of chronic lung disorders. It is of utmost importance that these different clinical phenotypes are taken into consideration when modelling COPD.

4.2 What are the major unmet needs to model and study COPD?

To date, inflammatory processes elicited by tobacco smoke have been widely studied in animal models [89-100]. Less well understood is how these inflammatory processes contribute to accelerated decline in lung function, how they persist following smoking cessation, and why they cause predominant airway versus parenchymal disease in subgroup of patients. Given the early origin of COPD [73], there is a need to study the contribution of lung development in the genesis of COPD. There is a further need for models of acute exacerbations [90, 94, 101-103] and COPD-associated comorbidities [92, 98, 104]. While these are important research areas, data today are still limited. For example, there is a limited understanding of inflammatory mechanisms involved in the various types of exacerbations of COPD [85] and how these processes contribute to the decline in lung function. A further

question that is currently not well understood is whether exposures to cigarette smoke, biomass fuel, and/or outdoor air pollution results in similar or differing pathogenetic mechanisms and disease phenotypes.

4.3 How can we model specific clinically relevant subgroups of COPD?

The pathogenesis of COPD is the result of a complex system of events perpetrated over decades; hence, experimental models represent the capacity to examine specific facets of the disease and study them in isolation from confounding factors. In addition, experimental studies have access to a broad range of reagents, tissues, and intervention strategies that are not available or feasible in clinical research. The strengths of these models however are also analogous to their weaknesses. The reductionist recreation of a subset of pathogenic components fails to recreate the complexity of the disease. This needs to be taken into consideration when translating observation from experimental models to the human disease [89].

4.3.1 Genetic factors, epigenetic alterations, and microRNAs

There is clear evidence that genetic and epigenetic factors influence the susceptibility for COPD. Of the genetic factors, alpha-1 antitrypsin (AAT) deficiency remains one of the most common risk factors for the development of emphysema [80]. While circulating AAT in specific mouse strains is associated with distinct patterns of emphysema [91], studying the loss of function of AAT in mice has been challenging, as there are multiple encoding genes (Serpina1a-1e) [105, 106]. Of interest is the report by Alam *et al.* that transgenic overexpression of the most common human mutant AAT in mice conferred a pro-inflammatory phenotype [107]. In addition to AAT, gene association studies have implicated numerous candidate genes over the past 40 years in the pathogenesis of COPD, although these studies have been largely inconclusive [108]. In more recent years, information was gained from applying genome-wide association (GWAS) to large cohorts [78]. While GWAS studies establish association, functional studies are required to gain insight into biological mechanisms of genetic determinants of COPD and/or lung function. For example, *in vitro* and *in vivo* experimentations have provided critical mechanistic insights into GWAS loci associated with COPD, such as HHIP and FAM13A [109-112]. These studies clearly document the importance of experimental models to study biological mechanisms and establish causality. Similarly, epigenetic modifications, as well as microRNAs (miRNAs) and long non-coding RNAs have been implicated in the pathogenesis of COPD [113, 114]. Moreover, there is some albeit limited evidence of epigenetic inheritance in COPD, i.e.

transmission of developmental programming across generations that were not exposed to the initial environment that triggered the effect [115]. Experimental models have and will continue to play a significant role in developing an understanding of the biological function of specific genetic factors, epigenetic alterations, and miRNAs in diseases processes associated with COPD.

4.3.2 Lung development

Emerging evidence suggests that factors in early life that affect lung development are implicated in the pathogenesis of COPD [73]. For example, two cohorts from mid-childhood to adulthood were assessed for their lung functions, and those with preterm birth and a history of bronchopulmonary dysplasia (BPD) had significant lower FEV_1 and mid-expiratory flow than term-born controls [116]. A longitudinal study also found an association between low quartile of maximal expiratory flow at birth and low FEV_1/FVC ratio at 26-32 years of age [117]. Moreover, intrauterine exposure to harmful stimuli (maternal smoke) and growth retardation, early childhood lung infection and asthmatic insults are all negatively affecting lung development and reducing the maximal FEV_1 in adulthood [118-121]. In addition, noxious factors encountered in early life may also accelerate the rate of FEV_1 decline in adult with active smoking [122].

Although growing evidence suggests early origins of COPD in humans, definitive and mechanistic studies are needed. Studies using animal models have already provided evidences regarding important developmental genes in the pathogenesis of COPD. For example, defective TGF-β-Smad3 pathway results in reduced alveolar growth during development that contributes to emphysema-like pathology in adult mice [123, 124]. Other key developmental pathways including FGF, Shh/HHIP, ErbB/ERRFI1, and VEGF have been shown to play key roles in both lung development and emphysema pathogenesis in genetically manipulated mice [109, 125-129]. Dysregulation of elastin fibre synthesis and degradation has been clearly demonstrated to affect both developmental alveolar growth and adult alveolar destruction [130, 131], one of the important mechanisms for COPD. In addition, there is evidence that prenatal exposure to cigarette smoke predisposes to airspace enlargement in adult mice, suggesting a role for *in utero* exposure on the adult development of COPD [132, 133]. Potential mechanisms include epigenetic modifications [134, 135], although further research is warranted.

4.3.3 Environmental factors

Cigarette smoke exposure models

Exposure of animals to cigarette smoke is perceived as one of the most relevant models to study smoking-associated lung pathologies [89-92, 94]. Of importance, human smoking behaviour varies substantially geographically and between individuals [136], and no single experimental smoke exposure system replicates the diversity of human smoking. Moreover, it is widely acknowledged that human smoking behaviour differs substantially from the commonly used Federal Trade Commission (FTC) and International Organization for Standardization (ISO) parameters [136]. As there are no standards available, smoke exposure parameters, such as mode (nose-only versus whole body exposure; side-stream smoke included or excluded), frequency, and duration vary profoundly between studies [93]. It is unsurprising that, in mice, the gene expression profiles differ markedly between studies [137]. It is likely that each experimental system reflects facets of the overall picture, given the diversity of human smoking behaviour. Complementing *in vivo* studies, the effects of cigarette smoke have been extensively modelled in *in vitro* systems using cigarette smoke extracts, cigarette smoke-conditioned medium, and air liquid interphase systems [89, 138]. Studies used cell lines and primary cells, as well as 3D culture systems and precision cut lung slices [89]. When using *in vitro* systems, levels of cigarette smoke exposure, type of cigarettes, and cell viability have to be taken into consideration and need to be reported to allow to compare studies.

Cigarette smoke exposure elicits lung inflammation in mice, rats, guinea pigs, sheep, and dogs [90, 91]. While cigarette smoke exposure of larger animals, such as rats and guinea pigs appear to elicit more robust airway response [90, 91], the mouse is the species that is most frequently used [93]. Advantages of the mouse model are the relative low cost, the rapid reproductive cycle and large litter sizes, as well as the vast number of molecular tools and the ability of gene manipulation with the induction of gain or loss of function [139]. In mice, inflammation is a function of the level of exposure to total particular matter based on studies by Hodge-Bell *et al* [140], using well-controlled exposure conditions. Hence, it is of critical importance that measures of exposure levels, such as total particular matter and cotinine levels are consistently provided in experimental studies.

While most animal studies are performed in young animals, COPD is a disease of the elderly. Schuster *et al.* reported that advanced age increased the susceptibility to cigarette smoke-induced pathophysiological hallmarks of COPD [141]. Contrasting these findings, no differences in airspace size were observed between mice exposed to cigarette smoke starting

at age 3 or 12 months [142]. Evidence suggests that women may have an increased risk of developing COPD compared to men, although the underlying mechanisms are not well understood. Tam *et al.* reported increased small airway wall remodelling that was associated with increased distal airway resistance in female compared to male mice [143, 144]. Clearly, further studies are warranted to assess how aging and gender impact inflammatory processes and tissue damage in animal models.

Biomass fuel exposure models

There is emerging evidence that exposure to indoor air pollution due to domestic use of solid biomass fuels (wood, dung, crop residue, charcoal) increases the risk of COPD [145, 146]. This has generated interest in models of biomass combustion-induced models of COPD. There are currently no comprehensive reviews how to model indoor biomass fuel exposure in animals. Current approaches include exposure of rats to wood burning smoke [147]. In this study, Hu and colleagues showed that biomass exposure elicited lung inflammation and airspace enlargement. Exposure of mice to wood or cow dung particulate matter collected from rural Indian homes during biomass cooking resulted in pro-inflammatory cytokine production, neutrophilic inflammation, airway resistance, and hyperresponsiveness [148]. More studies are required to establish models of biomass fuel-induced lung pathologies.

Air pollution

Although air pollution has been associated with worsening of symptoms in patients with COPD, the cellular and molecular processes are currently not well understood. Like cigarette smoke, air pollution is a complex mixture of particle matter and gases. The chemical composition is influenced by the production source, seasonal variations, and weather patterns [149, 150]. The gaseous components include among others, ozone, carbon dioxide, carbon monoxide, nitrogen oxides, volatile organic hydrocarbons. Particle matter is categorized by size into coarse particulate matter (PM10), fine particulate matter (PM2.5), and ultrafine particulate matter less than 0.1μM. Of note, the different sizes of particulate matter are able to induce different immune responses *in vitro* [151] and *in vivo* [152]. Further complicating modelling the adverse effect of air pollution is the fact that particulate matter component may act as a vehicle for biological components including endotoxin, pollen, and fungal spores, leading to complex multi-exposures [100, 153-155].

Air pollution models exist for mouse, rats, and guinea pigs and typically use particulate matter, gases (e.g. ozone), or a combination of the two factors (e.g. diesel exhaust) [90].

Exposure to air pollution leads to lung inflammation, goblet cell metaplasia, and lung function alterations. Evidence suggests that repeated exposure of mice to ozone may induce airspace enlargement [156, 157], although, this observation was not observed in other studies [158]. In this latter study, ozone had little effect on endpoints that were significantly affected by cigarette smoke exposure. Animal models of exposure to air pollution alone or in combination with cigarette smoke exposure will be valuable to explore how this environmental risk factor impacts the development and exacerbations of COPD.

Exposure to microbial agents.

Lipopolysaccharide (LPS), a glycolipid of the outer membrane of Gram-negative bacteria, is ubiquitously present as a contaminant in airborne particles, including air pollution, organic dusts, and cigarette smoke. LPS administration elicits robust neutrophilic inflammation [92]. Long-term LPS instillation in mice results in mucus cell metaplasia, airway wall thickening, airspace enlargement and altered lung function [159-162]. While this mimics persistent chronic inflammatory processes and tissue pathologies observed in COPD, glucocorticoids inhibit LPS-induced inflammation [163, 164]. This is in contrast to the steroid-insensitive nature of inflammatory processes that are associated with COPD [92].

4.3.4 Airway remodelling

In COPD, airway remodelling is characterized by thickening of the airway wall (including the smooth muscle layer), increased blood vessel density, enlargement of submucosal glands, mucus hypersecretion, and squamous and/or goblet cell metaplasia [165-168]. Airway remodelling is not a unique feature of COPD, but present in other chronic inflammatory lung diseases, such as asthma and cystic fibrosis. An important, but frequently neglected aspect of remodelling is that pathological remodelling has to be distinguished from physiological remodelling [167]. Physiological remodelling refers to structural changes characteristic of normal lung development and growth, as well as transient changes that occur during an acute response to injury and/or inflammation. In contrast, pathological airway remodelling refers to structural changes that result from disturbed lung development and growth, chronic injury, and/or inflammation that results in persistent altered airway wall structure.

Notably, radiologic studies indicated that terminal bronchioles are reduced by about 90% in lungs from patients with very severe COPD [169]. Moreover, these data suggest that destruction of terminal bronchioles begins long before the radiologic manifestation of emphysema. Notably, multivariate analysis suggested that remodelling of small airways

explained more of the variance in the association between structural changes and loss of function (FEV_1) than airway inflammation [170]. The vast majority of experimental studies in animal models have focussed on the pathogenesis and prevention of emphysematous lesions and only few approaches were made towards airway remodelling [171, 172]. Airway remodelling in rodent species exposed to cigarette smoke for a longer period of time typically presents as a mild form of small airway lesions. Loss of terminal bronchioles has never been reported in any exposure or genetic model. In general, the distinction between physiological and pathological remodelling is largely missing in studies of experimental animal models.

4.3.5 Alveolar destruction/emphysema

In human patients, emphysema is being considered as an end-stage phase in the progression of the disease. Most animal models have used cigarette smoke exposure. Only a few models have been using exposures related to air pollution particles, dusts, or biomass fuels [97, 173, 174]. Cigarette smoke exposure induces airspace enlargement in mice, rats, hamsters, and guinea pigs. Cigarette smoke-induced airspace enlargement is more robust in larger animals, such as rats and guinea pigs [93, 175]. It has been suggested that the development of emphysema in mice may go through different phases, with early repair and late failure to repair smoke-induced damage [176]. Hence, timing of intervention may need to be taken into consideration when applying observations from animal models to the human disease.

The use of transgenic mouse strains has helped to unravel mechanisms of cigarette smoke-induced emphysema [95-97]. Studies using these models have implicated protease/anti-protease balance, oxidants/anti-oxidants, apoptosis/proliferation, matrix destruction/deposition, pro-/anti-inflammatory mediators, accelerated aging, and autoimmune mechanisms in emphysema formation. At present, it is largely unclear whether the various mechanisms relate to physiological or pathological remodeling, which poses great difficulties on drawing the right conclusions. Remarkably, interventional studies designed to prevent or (better) treat experimental emphysema in animal models frequently reported positive outcomes, which is very much unlike clinical studies in human emphysema patients [97]. The reasons for this are currently not well understood. One aspect that may contribute is the fact that many studies use a single quantitative histopathological parameter to assess emphysema in animal models, i.e. mean linear intercept length, a parameter that has been controversially discussed [177]. Today, design-based stereology offers a number of structural parameters that allow for an unambiguous assessment of the loss of gas-exchange units in an experimental setting [178, 179].

In addition to cigarette smoke-induced emphysema, instillation of elastases, such as human neutrophil elastase and porcine pancreatic elastase, into the lungs leads to airspace enlargement [92, 96, 99]. Advantages of the elastase models are the rapid and robust on-set of the emphysematous lung destruction. Emphysema formation is accompanied by acute alveolitis, pulmonary edema, hemorrhage, and mucus cell metaplasia. Of note, inflammation in these models is transient only and does not reflect the progressive and slowly resolving inflammation associated with COPD [92]. Similarly, administration of a combination of LPS and elastase once a week for 4 weeks display hallmarks of COPD pathologies, including widespread lung inflammation, goblet cell metaplasia, increased lung volume, emphysema and decreased elastic recoil [180, 181].

4.3.6 COPD Exacerbations

Modelling acute exacerbations of COPD in animals has proven challenging due to the clinical and pathological complexity of the underlying disease and the fact that exacerbations have a variety of causes and severities [182]. Physiologically, acute exacerbations are characterised by worsening airflow obstruction and lung hyperinflation [183]. Patients that experience frequent exacerbations are now recognized as a distinct clinical subgroup, the 'frequent exacerbator' phenotype [82, 83]. To date, four distinct biologic clusters have been identified, including bacterial-, viral-, and eosinophilic-predominant exacerbations. The fourth cluster is termed pauci-inflammatory, as it is associated with limited changes in the inflammatory profile [85].

Viral and bacterial infections account for approximately 50-75% of all exacerbations [85, 184, 185]. Thus, understanding the underlying pathology of viral and bacterial infections and their consequences to lung function both in healthy and diseased lungs becomes increasingly important to study mechanisms associated with COPD exacerbations. For this reason, most animal models to date are based on models of viral or bacterial infection, and concurrent cigarette smoke exposure [90, 94, 101]. Importantly, disease severity is an important determinant of exacerbation frequency; hence, pathological changes characteristic of advanced disease, such as increased mucus production, thickening of the epithelium, or changes in epithelial cell integrity, and parenchymal damage may predispose to microbial infection [186]. While relevant, models that incorporate specific pathological features of COPD are less common. Finally, there are currently no models available for either eosinophil-predominant and pauci-inflammatory exacerbations.

Models of bacteria-predominant exacerbations

Bacteria most commonly associated with acute exacerbations of COPD include nontypeable *Haemophilus (H.) influenzae*, *Moraxella (M.) catarrhalis* and *Streptococcus (S.) pneumoniae*, while invasive bacteria such as *Pseudomonas (P.) aeruginosa* or *Chlamydia (C.) pneumonia* may be isolated in patients with more advanced disease [187, 188]. Numerous studies have examined the effect of cigarette smoke exposure on responses to bacteria [27, 189-193]. Evidence from several different laboratories suggests that cigarette smoke exacerbates inflammatory processes elicited by nontypeable *H. influenzae*. Of note, increased inflammation is associated with changes in lung function and tissue damage [27, 190, 191, 193, 194]. Similarly, cigarette smoke exacerbates inflammatory processes elicited by *S. pneumoniae* and *P. aeruginosa* [189, 191]. Whether increased inflammation affects physiological parameters, such as airflow and gas trapping is currently not understood. A further caveat of these studies is that the models utilize human pathogens in mice. Moreover, these models utilize instillation of single bacterial strains. This may be at variance with emerging data on the airway microbiome during acute exacerbations of COPD that highlight the microbial complexity associated with these events [187]. Clinically, there is a clear association between bacterial colonization and the frequency, character, and severity of COPD exacerbations [195]. Bacterial colonization has been an understudied aspect of COPD in animal models, primarily because it is difficult to select relevant microbial species [196]. Pathogens that colonize experimental animals often lack clinical applicability, while the clinically relevant pathogens often do not colonize animals. Importantly, bacterial colonization may impact the susceptibility to secondary viral and bacterial infections, or alter the ensuing immune-inflammatory responses, through mechanisms that are still poorly understood. This is an important knowledge gap as there is an association between bacterial colonization and exacerbation frequency.

Models of viral-predominant exacerbations

Human rhinoviruses (HRV) and respiratory syncytial virus (RSV) are responsible for the largest fraction of viral exacerbations [185, 197-199], although seasonal influenza has also been shown to be a significant cause of acute episodes. Most animal studies reported to-date have utilized influenza virus as the pathogen of choice to examine the consequences of cigarette smoke to antiviral host defense [16, 101, 103, 197], as influenza virus naturally cross infects diverse species. In contrast, HRV is highly species specific and does not infect rodents. This is because HRV uses the human intracellular adhesion molecule 1 (ICAM-1) to infect cells [200, 201]. The development of a transgenic mouse that expresses chimeric murine-

human ICAM-1 will facilitate studies examining the impact of cigarette smoke exposure on rhinovirus infection [202].

Cigarette smoke exposure exacerbates inflammatory processes elicited by influenza infection [203-205]. Increased inflammation is associated with increased tissue damage and airspace enlargement [204], providing experimental evidence that viral exacerbations contribute to disease progression. Cellular and molecular mechanisms that contribute to excessive inflammation are still actively investigated, but likely involve members of the IL-1 family, including IL-1 and IL-18 [17, 204]. Of interest is a recent study documenting that cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33 dependent response to infection [206]. The consequences of exacerbated inflammation to airflow limitation, gas exchange, and gas trapping are currently not understood. It is also unclear how the combination of cigarette smoke and viral infection affects cardiovascular parameters. Similar to bacterial exacerbation, current models use single viral agents. It is noteworthy that viral respiratory infections are often associated with bacterial pneumonia [207]. Moreover, approximately one third of exacerbations appear to be associated with both viruses and bacteria [185]. This raises a chicken and egg question: Does the viral infection predispose to bacterial superinfection or vice versa [208].

4.3.7 Comorbidities

Clinical evidence shows that COPD rarely occurs alone; the majority of patients with COPD are diagnosed with multiple comorbid conditions, with almost half of patients being diagnosed with four or more conditions [86]. Five comorbidity clusters were identified, including less comorbidity, cardiovascular, cachectic, metabolic, and psychological. Animal models may be used to study the pathogenesis of COPD-associated comorbidities [92, 98, 104]. For example, Apolipoprotein E (*Apoe*)-deficient mice, one of the most commonly used models in atherosclerosis research [209, 210], develop increased airspace enlargement in the lungs [211]. Lietz *et al.* showed that cigarette smoke exposure of *Apoe*-/- mice results in significantly increased aortic plaque size compared to room air exposed mice [212]. Cigarette smoke exposure of mice also led to skeletal muscle dysfunction and hypertension [92]. Hence, models of cigarette smoke exposure present an opportunity to investigate cellular and molecular mechanisms that contribute the development of cigarette smoking-associated comorbidities.

4.4 How suitable are the (current) COPD animal models to test and validate the efficacy of new drugs?

COPD is a complex condition, encompassing a spectrum of chronic lung disorders. While historically defined by progressive and poorly reversible airflow obstruction, emerging evidence suggests that both current and former smokers with preserved lung function may still present with respiratory symptoms and activity limitation [74]. Furthermore, it is estimated that almost half of the patients with COPD present with low FEV_1 in early adulthood, suggesting that perinatal and postnatal lung development may contribute to disease development [72]. No single animal model reflects this complexity. Data generated using experimental models have implicated protease/anti-protease balance, oxidants/anti-oxidants, apoptosis/proliferation, matrix destruction/deposition, pro-/anti-inflammatory mediators, lung development (early origin), accelerated aging, and autoimmune mechanisms in the pathogenesis of COPD. Similarly, animal models of viral or bacterial infection, and concurrent cigarette smoke exposure have contributed to our understanding of mechanisms of COPD exacerbations [90, 94, 101-103]. Animal models have also been used to study COPD-associated comorbidities [92, 98, 104]. Thus, although experimental models have provided insight into putative pathogenetic mechanisms associated with COPD, successful translation of these observations from bench to bedside will require a clear understanding of the relevance of specific experimental models relative to specific COPD phenotypes. Hence, careful selection of specific model systems is indicated to model aspects of the overall disease. This requires an in-depth understanding of the research question, the strengths and limitations of the corresponding models, and selection of clinically relevant endpoints. Drugs developed using this approach will likely not be suitable for all patients with COPD, but be effective in well-defined patient subpopulations.

1) No single experimental model reflects the overall complexity of COPD.
2) Current experimental models can be used to study specific clinical phenotypes.
3) Modelling COPD requires in-depth understanding of the research question, the strengths and limitations of the corresponding models, and selection of clinically relevant endpoints.

5) Infection/Pneumonia

5.1 Introduction

Clinically, lung infections are induced by viable pathogens that spread and replicate in the lungs and should be distinguished from pathogen colonization of the lung and/or airways. Pneumonia frequently involves systemic inflammation with distant organ pathology, which may be related to bacteremia and systemic inflammatory effects of bacteria and/or with local pulmonary inflammation and spillover of inflammatory mediators. *Streptococcus pneumoniae* (*S. pneumoniae*, the pneumococcus) is the most prevalent pathogen in community-acquired pneumonia (CAP) in humans [213-216], accounting for more deaths in humans worldwide than any other single pathogen [217]. Pneumococci may experimentally cause pneumonia not only in mice and rats, but also in various other mammals, including guinea pigs, dogs, cats, horses, gorillas, and dolphins [218]. A considerable portion of our current knowledge about the dynamic host-pathogen interaction in bacterial pneumonia stems from research involving this pathogen. However, many other pathogens, including bacteria, viruses and fungi also cause pneumonia and have been investigated in experimental pneumonia models.

Besides living pathogens, specific pathogen products, the so-called pathogen-associated molecular patterns (PAMPs) stimulate pattern recognition receptors (PRRs), thereby inducing inflammatory reactions and pathologies in the lung that partly reflect lung infections. However, the dynamics of the PAMP-induced processes usually differ substantially from lung infections.

We briefly summarize advantages and shortcomings of current experimental pneumonia models, focusing on unmet needs, requirements for meaningful experimental protocols and endpoints regarding therapeutic interventions and the importance of lung-distant organ involvement.

5.2 How is (infectious) pneumonia defined and what do experimental models need to reflect?

Pneumonia is an acute infection of the lung parenchyma caused by a pathogen. The most common pathogen in bacterial community-acquired pneumonia (CAP) is *Streptococcus pneumoniae* (*S. pneumoniae*), followed by *Mycoplasma pneumoniae*, *Legionella* spp., *Haemophilus influenzae*, *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*),

Klebsiella spp. and others. The most frequent pathogens in bacterial hospital-acquired pneumonia (HAP) are *Pseudomonas aeruginosa*, *S. aureus*, *Acinetobacter* spp., *Serratia*, *Proteus* spp., *E. coli* and *Klebsiella* spp. Further, viruses (e.g. *influenza*, *respiratory syncytial virus*) and fungi (e.g. *Candida* spp., *Aspergillus niger*) are causative organisms of pneumonia. In immunocompromised individuals, pneumonia may also be caused by tuberculous and non-tuberculous *Mycobacteria*, *Pneumocystis jirovecii*, *Nocardia* spp. and *Rhodococcus* spp. or others [219-222].

The pathogen, and also the serotype determine the lung phenotype after infection. More than 90 serotypes of *S. pneumoniae* have been identified so far [223], with major differences in virulence profiles among and between serotypes. For example, while some pneumococcal serotypes such as types 19, 23, and 33 are less invasive in mice and humans and primarily affect patients >70 years with underlying co-morbidities, other serotypes of *S. pneumoniae* such as types 2, 3, 4, or 7F are naturally more invasive, causing bacteremia and invasive pneumococcal disease (IPD) even in younger, immunocompetent patients as well as in mice [224-226]. Therefore, in pulmonary infection research, the chosen serotype often defines the developing disease entity.

The most widely employed animal model system of bacterial pneumonia makes use of laboratory inbred mice, as well as rats, since many features of bacterial pneumonia are common in humans and rodents, such as early secreted proinflammatory cytokines, as well as the ensuing bronchoalveolar recruitment and activation of inflammatory leukocytes, including interstitial and alveolar recruited neutrophils, exudate macrophages, and a delayed lymphocytic response [227-230]. Lung inflammatory responses to bacterial challenge can, at least in part, be mimicked in mice by application of purified bacterial toxins such as lipopolysaccharide from Gram-negative *E. coli* [227], or pneumolysin from Gram-positive *S. pneumoniae* [231, 232], or even in part by defined upstream cytokines, such as Tumor necrosis factor-α (TNF-α) or Interleukin-1β (IL-1β) [233, 234]. Histopathological hallmarks of bacterial pneumonia in mice and pneumonia patients include interstitial and alveolar fibrinous exudates along with a characteristic neutrophilic alveolitis, which is usually diagnosed by bronchoalveolar lavage [228], as well as purulent bronchopneumonia with or without the histologic pattern of consolidating pneumonia. Without any need of host adaptation, clinical isolates of bacterial pathogens can be employed to study the dynamic host-pathogen interaction in mice, while a major drawback of most viral mouse infection

models is the need to use mouse-adapted viruses in order to achieve productive infection and clinically apparent signs of disease [235]. Numerous transgenic, knockout and knock-in mice as well as mouse-specific immunological tools are commercially available. Since laboratory mice are relatively cheap and easy to handle, mice have become the most popular animal model system in lung bacterial infection research to date.

Major differences between human and murine bacterial pneumonia need to be taken into account. As one example, major species-specific differences exist in the kinetics of resolving bacterial pneumonia: Empirical observations suggest that the overall clearance of pneumonic infiltrates in patients with e.g., pneumococcal pneumonia may take between two to four weeks, depending on the severity of the disease, with a considerable portion of such patients being re-hospitalized in the US within 30 days [236], whereas histological examinations in mice with pneumococcal pneumonia demonstrated substantially accelerated kinetics of resolving pneumonia in the range of 5-10 days [237, 238], with obvious relevance for translational infection research.

Several techniques are available to deliver pathogens into the lungs of mice, like intranasal instillation, oropharyngeal intubation of mice followed by intratracheal aspiration, and surgical exposure of the trachea followed by direct injection of bacteria into the tracheal lumen for subsequent fluid aspiration:

- *Intranasal application* of bacteria can either be used to establish nasopharyngeal colonization of mice, or to induce bacterial pneumonia in mice, which is largely dependent on the fluid volume applied [239, 240]. In case bacterial pneumonia is induced via intranasal routes, this application will inevitably also cause nasopharyngeal pathogen recognition, which depending on the immune status of the mouse, may possibly impact the disease course of pneumonia. Generally, it requires lightly anesthetized mice and, for safety reasons, is recommended to be performed under a laminar flow hood.
- *Intratracheal instillation* of bacteria into the lungs of mice can be achieved by previous oropharyngeal intubation of a vertically fixed and anesthetized mouse using a teflon catheter under visual control of the pharynx via illumination of the mouse neck, and represents a fast and non-invasive as well as secure way of bacterial administration to lung distal airways [224, 225, 237, 241, 242]. Care must be taken to correctly intubate the mouse, before bacteria are instilled. Using this method of lung

bacterial infection, the applied volume should be limited to 50 µl per mouse lung, which is well tolerated, but should be applied in small aliquots and not as a bolus. Filling the syringe with 500 µl of air prior to filling in the bacterial suspension will allow the investigator to completely empty the syringe as the last aliquot is being delivered into the lungs. Alternatively, a MicroSprayer™ can be employed, which enhances homogeneity of fluid distribution in the lung periphery [243-245]. For infection of one specific lung area (i.e. lobe, segment), miniaturized bronchoscopy can be used [246].

- The third maneuver to deliver bacteria into the lungs of mice involves *surgical exposure* of the trachea of an anesthetized mouse, which is fixed in a horizontal position. The bacterial suspension is injected directly into the trachea [231, 247]. The advantage of this technique is that the fluid aspiration process itself can be observed easily under a stereo-microscope, which may be advantageous when oropharyngeal aspiration is less well tolerated by a given mouse strain, or when using very young mice. The disadvantage of this application route is that it requires a surgical preparation of the trachea, and therefore is more time consuming and invasive.

5.3 What are the major unmet needs to model and study pneumonia?

The lungs contain an uncounted number of different cell types; the respiratory epithelium alone may contain nearly 50 different cell types [248]. For the modelling of human diseases, cells in culture need to represent their *in situ* counterparts in appearance, gene expression, functions and the signaling pathways related to these functions. That cells lose many properties and functions when removed from their native site has long been known [249] and the crucial question is whether these cells retain enough functions to make them suitable to study at least some aspects of complex diseases. Major problems in using cultured cells to study diseases come from problems with phenotypic representation and stability.

Phenotypic Representation.

To know whether the *in vitro* system in question represents the *in situ* situation, we need at least a gross understanding of the physiological blueprint, which however in the lungs is far from trivial: (i) The properties of many cells depend on their exact location; for example, the properties of pulmonary endothelial cells and of bronchial epithelial cells change gradually along their way. (ii) Even similar-looking cells from the very same region may have different functions as is suggested by the presence of endothelial pacemaker cells [250] or

heterogeneous epithelial cell populations. (iii) The phenotype and the properties of individual cells *in situ* may be more fluid than was once believed [251].

Phenotypic Stability.

Once the representative cell has been taken into culture, it must retain its phenotype. However, the plasticity of many seemingly terminally differentiated cells may be much greater than once thought possible. For instance, conversions of fibroblasts into skeletal muscle cells [252] or of B-cells into macrophages [253] have been described and the dedifferentiation of cells requires only a few transcription factors [254]. In the lungs it was shown that FGF can reprogram tracheal epithelium into distant lung cells [255]. In addition there is compelling evidence that primary pulmonary epithelial and endothelial cells change their gene and protein expression patterns shortly after their removal from the lungs [256, 257]. Therefore, it appears likely that there is a multitude of cues from neighboring cells, from mediators and from the prevailing physical forces that constantly induce or maintain a given phenotype.

From this it becomes obvious that the culture of pulmonary cells is fraught with many serious problems. Hence, it may not be surprising that in those studies that cells in culture systematically compared with their real counterparts, the degree of resemblance was found to be at best faint. Pulmonary epithelial type I and type II cells taken into culture changed their phenoytpe within 6 hours according to gene expression studies [257]. In a study on the usefulness of *in vitro* models to predict lung toxicity, human airway 3D models or A549 cells did not respond different than 3T3 cells, and none of them had strong predictive power [258]. Confluent endothelial cells in culture that are frequently used to study pulmonary edema respond in many ways different than endothelial cells in the lungs: cells in culture, for instance, have untypical calcium and NO levels and dynamics, they respond to mediators (e.g. thrombin, LPS, TNF) that do not cause pulmonary edema directly (they can of course do so indirectly, through the activation of leukocytes) and they do so by mechanisms that up to date do not appear relevant in intact lungs (e.g. Rho kinase) [259]. These comprehensive studies clearly demonstrate that it is not sufficient when cultured cells resemble their counterparts in terms of provenience, look and the expression of some genes, but that functional resemblance is also critical. For meaningful models, the addition of infectious agents to cells in vitro even further increases the complexity of requirements.

Therefore, at present there is little comprehensive evidence for any parenchymal lung cell that would suggest that these cells retain in culture enough properties to give them a high predictive power for the *in situ* situation. The current massive interest in 3D models [260, 261], microfluidic systems [262, 263] and mechanical factors [263-265] of cultured pulmonary cells indicate that these insufficiencies have been recognized and that there is some hope that these approaches will provide us with better *in vitro* tools. Because usually the contact with other cells dramatically alters the behavior of cells [266-268], it may well be that 3D-printing techniques [269] will be required to approach the *in vivo* situation.

In our opinion, progress in this area also depends on a much deeper understanding of the programming of lung cells, including epigenetics. The scope of the problem is further illustrated by findings from developmental biology where it is known that the same factor (e.g. FGF) can even have opposite effects depending on timing, tissue site, and perhaps its level of signaling [255]. Taken together, the current technologies for culturing lung cells bear so many problems that at present caution needs to be advised when extrapolating *in vitro* results to the *in vivo* situation.

Intrinsic host susceptibility

Intrinsic host susceptibility to infection also needs to be taken into account, which is influenced by gender, age and existing co-morbidities [270, 271]. Studies in mice have revealed a great diversity in terms of infection susceptibility between most commonly employed mouse strains [272, 273]. For example, C57BL/6 mice (favoring Th1 responses) and BALB/c mice (favoring Th2 responses) were found to be more resistant, while DBA/2 and 129S2 mice were found to be more susceptible to challenge with either bacterial or mycobacterial or viral pathogens [272-277]. After pneumococcal infection (D39) of nine inbred mice strains, Gingles et al. observed a susceptible phenotype (CBA/Ca mice and SJL mice) and a resistant phenotype (BALB/c mice) and suggest an association between susceptibility or resistance and recruitment and/or function of neutrophils [278].

The aspect of host susceptibility to infection must be considered when studying bacterial (or any other) infections *in vivo*, or when infection studies using different mouse strains are compared with each other. Using a highly genetically-diverse mouse resource population, the so-called Collaborative Cross (CC) mice, a recent study suggested that particularly the host genetic background defined the risk of morbidity and mortality in a model of *P. aeruginosa*

pneumonia, whereas initial variables such as body weight, age and gender had only a limited influence on outcome [276]. Nevertheless, despite this important finding, appropriate design of an experimental infection study means to exclude as much as possible any variables such as gender and age, as well as differences in the genetic background that might affect readouts. Actually, most researchers use female mice at the age of 8-12 weeks, rather than males, which however is most probably simply due to practical rather than scientific considerations, as males exhibit a more aggressive group behavior compared to females, which in turn makes their housing more difficult and expensive. However, various reports demonstrate gender differences in susceptibility to CAP in humans as well as in experimental infection models in mice: For example, prospective studies from Spain reported higher incidence rates of CAP in males as compared to females, with a significantly increasing incidence of CAP with ageing >75 years [279]. As a possible explanation, a recent report suggested that enhanced pneumococcal killing by alveolar macrophages from female mice and humans, and improved survival of pneumococcal pneumonia in females as compared to males was due to estrogen-mediated activation of lung macrophage nitric oxide synthase-3 (NOS3) [280]. Such gender differences in host infection susceptibility imply that results obtained from infection studies employing females may not necessarily be applicable to males.

Microbiota

Finally, the microbiota which is influenced by environmental factors (e.g. husbandry practices) and its significant impact on the outcome of animal experiments [281] has to be considered. Ma et al. investigated the effects of changes in common husbandry practices (food bedding, facility, cage) on the gut microbiota over a short time course of five days [282]. They found a transient change in microbiota after a short cross-campus facility transfer, but did not detect comparable microbiota alterations due to changes in common laboratory food or bedding, or following an isolated cage change in mice acclimated to their housing facility. These results highlight the importance of the acclimation period following transfer of mice. Zhang et al. demonstrated that diet has a dominating role in shaping gut microbiota. They showed that changes of some key populations may transform the gut microbiota of wildtype mice into a pathogen-like entity relevant to development of metabolic syndromes, despite a complete host genome [283].

There is emerging evidence that gene deficiencies influence the gut microbiota leading to changes in microbiota composition; this influence was demonstrated by numerous studies for genes involved in host response to pathogen-associated molecular patterns such as TLR and

NOD family members [284-286]. In conclusion, controlling basic animal husbandry practices is crucial to keep the influence of the gut microbiota on the course of pneumonia as low as possible.

While there is increasing evidence for a lung microbiome, its impact on invading pathogens and pulmonary immune responses in infections needs to be identified.

5.4 How can we model specific clinically relevant phenotypes (e.g. local vs. systemic infection) in pneumonia?

Significant experimental research has been done focusing on *Streptococcus pneumoniae*, the major bacterial agent of community-acquired pneumonia. The disease entity in mice following transnasal pneumococcal inoculation is defined by the chosen serotype, thereby allowing modeling of different clinically relevant phenotypes. While infection of mice with *S. pneumoniae* serotype 2 (D39) leads to a primary sepsis-like disease, mice challenged with serotype 3 pneumococci develop pneumonia with subsequent bacteremia [287]. If left untreated, mice infected with *S. pneumoniae* serotype 3 develop lethal ARDS and sepsis within 72h post infection [288]. Interestingly, reducing the pneumococcal infection dose does not lead to a mild, self-limited course of pneumonia (unpublished data). Using an LD50, two distinct clinical courses in infected mice are observed, sick and non-sick animals. But, infecting mice with a lethal dose of *S. pneumoniae* (PN36) and treating those when severe pneumonia was established (24h after infection) with antibiotics to kill pathogens, enables monitoring of the resolution of infection-induced inflammatory processes [288].

Cardiac and vascular complications are commonly observed in CAP patients and substantially contribute to the mortality of hospitalized older patients [289]. Recently it was shown that *S. pneumoniae* is capable to translocate across the vascular endothelium into the myocardium and form bacteria-filled microlesions, which may contribute to heart failure during fulminant invasive pneumococcal disease [290]. To address specific aspects of pneumococcal-induced microlesion formation in the heart, detailed protocols for the mouse model were recently published [291].

Mechanical ventilation is the only live-saving intervention in patients with acute respiratory failure (due to pneumonia), although it may cause ventilator-induced lung injury (VILI). This clinical scenario can also be studied experimentally in a second-hit model of established

pneumococcal pneumonia and mechanical ventilation. In this model it was demonstrated that mechanical ventilation with moderate tidal volumes aggravated lung injury and promoted progression of sepsis and multiple organ failure in pneumococcal pneumonia [292].

5.5 How suitable are the currently available models to test and validate the efficacy of new drugs in pneumonia?

Testing new therapeutic approaches (antibiotics, adjunctive therapies) requires meaningful experimental protocols that match the clinical setting as good as possible. There are some key aspects that need to be considered when studying new treatments. Adjunctive therapies should be tested in infection models with antibiotic treatment to match the clinical setting. Therapeutic administration of substances should begin after disease symptoms are established and frequency adapted to the metabolism of the subject employed for the experiment. It should be noted that pharmaco- and toxicokinetics of substances differ between animals and humans [293]. Adaptation to the metabolism of the subject employed for the experiment is necessary, taking substantial changes of metabolism in infected or even septic animals into account. The application route should be selected according to the properties of the substance to be tested and the objective of the study. For this, knowledge about the chemical and physical characteristics of the substance is mandatory [294]. Further, volume of administration (may alter course of disease) and site of delivery (e.g. resorption from gut or peritoneum or subcutaneum may be changed due to severe illness) have to be considered [295]. When substances are applied as solutions/suspensions, vehicles should be carefully selected to prevent unintentional adverse effects on the animals or disease course [296].

Determining the efficiency of a new treatment requires meaningful endpoints. Currently, the most valid (common) way is to measure the (approximate) survival of the infected animals following treatment. There are attempts to identify reliable predictors of severity and outcome of the infectious disease (earlier, more humane endpoints) to reduce pain/distress of the animals. “The earlier endpoints should be based on scientifically valid values of variables, not just honest judgments based on an appreciation of the distress and pain the animal may be experiencing. For example, animals becoming sick in an infection challenge or an antibiotic efficacy trial might be euthanized for humane reasons when in fact they could have survived the challenge, proving that a (new) antibiotic was an effective treatment.” [297]. Body temperature decrease (e.g. more than 4-6°C) has been shown to indicate deterioration in animals’ condition in infection experiments [298-300]. Bast et al. demonstrated that changes in skin temperature (measured by infrared temperature scanners) are predictive of mortality in

a murine model of pneumonia that was used to evaluate drug efficiency. Therefore, the authors suggested that skin temperature might be used as an earlier more humane endpoint than death without affecting scientific concerns [301]. Olfert et al. recommend using a predefined amount of weight loss (e.g. 10-20% or 20%) as well as its duration and consistency as an endpoint for infectious disease animal models [297]. Overall, regular clinical monitoring including measurements of body weight and rectal temperature according to an approved pain and distress scoring system with clearly predefined humane endpoints minimizes pain and distress of the animals.

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