Theme VI – Human Relevance

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Session VI-1a: *In vitro* disease models – Lung

Co-chairs
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Session VI-1a: Oral presentations

VI-1a-162

Efficacy assessment of novel anti-viral, anti-inflammatory and mucolytic agents using human airway epithelium (MucilAir™)

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The human airway epithelial cells play crucial roles in the pathogenesis of important respiratory diseases such as asthma, cystic fibrosis (CF), chronic obstructive pulmonary diseases (COPD) (Huang et al., 2009). Furthermore, due to the particular tropism of bacteria and viruses, sometimes, only human cell/tissue models are suitable for studying these human pathogens and for anti-viral and anti-bacterial drug development (Huang et al., 2011).

Using MucilAir™ , a standardized human airway epithelium model both morphologically and functionally fully differentiated which can be maintained at a homeostatic state for more than a year, the following tests have been developed:

Anti-inflammatory: Based on Il-8 release, this assay allows ranking anti-inflammatory drugs upon challenge with LPS.

Anti-viral agents: human Rhinoviruses such as the type-C, extremely difficult to grow on other cell models, infect and replicate efficiently in MucilAir™ ; the replication is almost completely inhibited by novel antivirals agents such as Rupintrivir. These results demonstrate that MucilAir™ is a reliable and powerful tool for anti-viral and anti-bacterial drug development (Tapparel et al., 2013).

Mucolytic agents: using cilia beating frequency measurement, mucus secretion and mucociliary clearance as end-points, the effect of reference compounds and comparative study between different pathologies (COPD vs CF vs Normal donors) has been evaluated.

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VI-1a-442 *

IL-13 induced asthma model in human precision-cut lung slices

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Novel therapeutic treatments are required for patients suffering from chronic respiratory diseases. Rodent model reflect some features of allergic asthma, however, human physiology is missing. To reduce animal testing and to overcome limitation of rodent models human precision-cut lung slices (PCLS) were chosen as an *ex vivo* tissue model. To mimic features of allergic asthma, e.g., airway immune response, mucus hypersecretion and airway hyperresponsiveness, human PCLS were stimulated with interleukin (IL)-13.

PCLS were prepared from human lungs and incubated with IL-13 for induction of inflammation, mucus production as determined by ELISA. IL-13-induced bronchoconstriction was measured after methacholine (MCh) provocation and visualized by videomicroscopy. Specificity was proven by usage of IL-13 antagonists.

Human IL-13 induced the secretion of eotaxin-3 and TARC. Both cytokine were blocked by addition of inhibitors (anti-IL-13 or anti-IL-4Rα chain). Human IL-13 induced mucus hypersecretion (2-fold compared to control) in bronchial tissue. Strikingly, airway hyperreactivity was induced here demonstrated by decreasing EC_{50} values for MCh from 180 nM to 47 nM and by an increase in maximal bronchoconstriction.

This study shows that human tissue mimics features of airway immune response, mucus hypersecretion and airway hyperreactivity, which can be used for drug development and preclinical testing.

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VI-1a-466

Ex vivo models for asthma, COPD and lung injury in precision cut lung slices (PCLS)

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Research from basic science has remarkably changed the general perception of 3D organotypic tissue models. Precision cut lung slices (PCLS) display such a suitable *ex vivo* tissue model that maintains microanatomy and functionality of the respiratory tract. The model allows the investigation of effects of compounds and drugs directly on cytokine release and functional responses such as bronchoconstriction under similar experimental conditions in different species including human. The tissue can be stimulated with, e.g., chemicals, lipopolysaccharides, bronchoconstricting agents and disease-related proteins. Depending on the underlying immunology, lipopolysaccharides and proteins such as IL-13 induce an acute increase of pro-inflammatory cytokines and/or airway hyperresponsiveness. Effects of chemicals were shown to correlate with *in vivo* inhalation toxicity studies. We found that the tissue response is highly comparable with the *in vivo* response. In summary, PCLS can be used as model to study several features of lung injury, COPD and asthma *ex vivo*. The different tissue responses can be used for the prediction of toxicological endpoints and adverse health outcomes such as organ injury, respiratory sensitization and inflammation. The presentation will give an overview about the current use of lung tissue in inhalation toxicity but also state their use for drug research.

VI-1a-740

Responses of *in vitro* asthmatic human airway epithelial cultures to rhinovirus and poly(I:C)

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Human rhinovirus (HRV) is a common cold pathogen and inducer of asthma exacerbations. Human airway epithelial (HAE) cells are targets of HRV infection. To gain increased understanding of mechanisms linking HRV infection to asthma, we compared the response of submerged and ALI cultures to HRV, the effects of HRV and Poly(I:C) in HAE cultures, and evaluated differences in HAEs from asthmatic and non-asthmatics responses to HRV. Cultures from 6 asthmatic and 6 non-asthmatic donors were exposed to HRV or Poly(I:C). Gene expression and cytokine/chemokine secretion were determined at 1.5, 8, 24 and 48 h post-exposure. Genome-wide gene expression changes were analyzed using RNA-seq technology. HRV and Poly(I:C) induced significant responses in gene expression and cytokine/chemokine secretion. Responses were dependent upon culture condition and exposure agent. Poly(I:C) elicited stronger responses in submerged cultures, but was less effective in eliciting effects from ALI cultures. HRV elicited stronger responses in ALI cultures, but was less effective in eliciting effects from submerged cultures. Compared to other models, ALI culture systems challenged with HRV better recapitulate *in vivo* responses to viral infection. Evidence of differential expression in asthmatic vs. non-asthmatics was shown. This *in vitro* model may facilitate understanding of viral exacerbation mechanisms.

VI-1a-900

Intravital imaging of human lung tissue for simulation of severe lung infections

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Worldwide pneumonia is the most frequent cause of death due to infections and mortality rates remain constant since decades. Therefore, new therapeutic options for immune-modulation to control destructive hyper-inflammation are needed. However, the overwhelming majority of scientific data is based on mouse models and molecular knowledge of human pathogen-host-interactions is underrepresented.

Therefore, we established a human lung tissue culture model to investigate infectious processes directly in the human alveolar compartment.

A great advantage of the model is that human pathogenic bacteria and viruses can now be investigated which are unsuitable in animal models. Next to determination of replication rates, cellular targeting and tropism as well as immune factor regulation we established overexpression of fluorescence coupled proteins in the tissue and by use of spectral intravital imaging functional molecular microscopy such as FRET/FRAP became possible.

Our recent publications, e.g., contributed to the understanding of alveolar type II cells during infection, showed the cellular replications niches of different types of Influenza A or even the alveolar damage of emerging SARS-like MERS-corona virus (Hocke et al., 2013a,b; Knepper et al., 2013; Szymanski et al., 2012; Weinheimer et al., 2012).

Taken together, the use of human (lung) tissue can serve for the simulation of human diseases and their analyses on a high technical level thereby allowing for the replacement of animal experimentation for the addressed hypotheses.

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Session VI-1b: *In vitro* disease models – Infection

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Session VI-1b: Oral presentations

VI-1b-393

In vitro triple culture of inflamed human intestine as a model to investigate nanoparticle safety and efficacy

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Nanoparticles offer several advantages to improve drug delivery to the intended site of action, which is promising for inflammatory bowel disease. Nevertheless the interactions between nanoparticles and biological systems might be an important issue in disease conditions. Therefore, advanced and reproducible *in vitro* models that mimic intestinal inflammation are valuable as alternative model to animal tests (Leonard et al., 2010).Here we improved the 3D model of human inflamed intestine (Leonard et al., 2010), by using cell lines to have a more robust and reproducible system to assess particle toxicity and drug delivery efficacy.

THP-1 and MUTZ-3 were embedded in a collagen layer in transwell filter, with Caco-2 cells cultivated on top. This model was inflamed with IL-1β and particle toxicity and anti-inflammatory efficacy assessed through pathophysiological changes (epithelial barrier function and pro-inflammatory cytokines release). Au, TiO_2 and Ag particles, relevant to oral exposure, showed more realistic results regarding toxicity and inflammation in the 3D culture compared to the monoculture. Anti-inflammatory effect on 3D inflamed culture was reached with PLGA based nanoparticles and microparticles loaded with Cyclosporine A. Thus, the present model of inflamed human intestine is valuable to evaluate cytotoxicity and efficacy of engineered nanomaterials relevant to oral exposure as well as drug delivery efficacy.

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VI-1b-525

Regulation of pro- and anti-inflammatory human Th17 cell properties

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Th17 cells have emerged as a new T helper cell lineage involved in the clearance of extracellular bacteria and fungi. A dys-regulated Th17 response, however, can induce severe tissue destruction and autoimmunity. Therefore, mechanisms must be in place to shield the host from immune-mediateddamage.

We demonstrate that human Th17 cells transiently produce the anti-inflammatory cytokine IL-10 upon stimulation. Interestingly, IL-10 expression was accompanied by reciprocal down-regulation of IL-17, leading to a functional regulatory Th17 cell phenotype after the peak of the effector response. The ability of Th17 cells to express IL-10 was, however, restricted to certain antigen specificities. *Ex vivo* isolated *C. albicans* specific Th17 cells could not produce IL-10 in comparison to *S. aureus* specific Th17 cells. This was due to differential priming requirements of these Th17 cell sub-populations. IL-1beta instructed naïve T cells to develop into a pro-inflammatory non-IL10 expressing Th17 cell subset. Th17 cell priming with *S. aureus*, however, was not IL-1beta dependent, leading instead to the generation of IL-10 producing Th17 cells with self-regulatory activities. This approach revealed that IL-1beta is a molecular switch for determining a pro- versus anti-inflammatory Th17 cell functions.

VI-1b-581

Vitamin D-dependent antimicrobial pathways in human macrophages

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T cell-mediated activation of macrophages is crucial for effective control of intracellular pathogens, such as mycobacteria. However, the mechanisms by which human macrophages kill intracellular pathogens are not well understood. We investigated the T cell-mediated activation of macrophage antimicrobial responses by studying primary immune cells isolated from human skin and blood. We found that activation of macrophages by T cell IFN-γ and/or CD40 ligand induces an anti-mycobacterial pathway dependent on the intracellular conversion of 25-hydroxyviamin D (25D) into the bioactive 1,25-dihydroxyvitamin D (1,25D). 1,25D mediated activation of the vitamin D receptor results in the induction of antimicrobial peptide expression, as well as autophagy, which is required to overcome the phagosome maturation block in infected macrophages. Of relevance, vitamin D production is dependent on exposure to UV light and reduced in dark-skinned populations. In our model, sera from white individuals with sufficient amounts of 25D, but not sera from African-Americans with lower 25D levels, support the vitamin D host defense response. In summary, we could show that T cells activate a vitamin D-dependent antimicrobial activity in human macrophages and that cutaneous vitamin D synthesis and host defense pathways are possibly linked in humans.

VI-1b-639

A human *in vitro* allergy model showing allergen specific immune responses using house dust mite or grass pollen allergen

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Rationale: In the study described, a human *in vitro* allergy model was established providing an alternative method to animal use investigating allergic immune responses. The model should be able to show the influence of cellular or drug candidates avoiding animal experiments and show close relation to the human situation.

Methods: Antigen-presenting cells were generated from whole blood of healthy or allergic donors. APC were stimulated with house dust mite extract (HDM) or Phlp5 and co-cultured with autologous CD4+ lymphocytes in the presence of allergen and or immunomodulatory compounds. Effects on T cell proliferation and cytokine secretion were analyzed by ^{3}H-thymidine incorporation and ELISA or Multiplex analysis.

Results: Allergen-pulsed APC induced strong proliferation of the CD4+ lymphocytes. The presence of immunomodulatory plasmacytoid DC (pDC) in the cocultures inhibited this response by about 50%. Proliferation of HDM-stimulated PBMC was dose-dependently reduced by pDC showing a significant deregulating functionality.

Conclusions: Allergen-specific T cell response can reproducibly be measured using the human *in vitro* allergy model showing regulatory properties of pDC. This model mimics regulatory mechanisms of allergic immune responses and might be applicable for efficacy testing of immunoregulatory cellular therapeutics or biomolecules with greater predictivity for the human situation and avoiding animal experiments.

Session VI-1c: *In vitro* disease models – Skin

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Session VI-1c: Oral presentations

VI-1c-124

Modeling hypersensitivity of human skin towards UV radiation from the DNA-repair deficient genetic syndrome xeroderma pigmentosum

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Sun exposure has been clearly implicated in premature skin aging and cancer development. The deleterious effects of UV radiation are exacerbated in patients with xeroderma pigmentosum (XP), a rare genetic disease caused by a deficiency in nucleotide excision repair (NER) of UV-induced mutagenic lesions. XP cells therefore represent a relevant model to study sequences of events leading to skin photoaging and photocarcinogenesis.

Using keratinocytes and fibroblasts isolated from small skin biopsies of XP-C patients, the first XP-C organotypic skin cultures were generated (Bernerd et al., 2001). They could reproduce the NER defect in situ after UVB exposure and revealed yet undescribed defects in epidermal differentiation. Moreover, XP-C skin reconstructs unveiled the pro-invasive role of XP-C dermal fibroblasts, leading to the formation of epidermal invasions resembling early steps of neoplasia. Further characterization of XP-C fibroblasts revealed a photoaged-like phenotype, including an overexpression of MMP1 and a higher content of intracellular ROS (Frechet et al., 2008).

Reconstruction of XP skin *in vitro* thus represents a valuable three-dimensional model to study the impact of UV radiation in a context of photosensitivity. Furthermore, the possibility to reconstruct genetically corrected XP-C skins with restoration of DNA repair and cell survival after UV exposure now opens perspectives for therapeutic approaches (Warrick et al., 2012).

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VI-1c-282

PPAR agonists do not exhibit their beneficial effects in inflammatory skin diseases by upregulating FLG expression

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Loss-of-function mutations in the filaggrin gene (FLG) are the major predisposing factor for atopic dermatitis (AD). Peroxisome prolifer-

ator-activated receptor (PPAR) agonists are known to increase FLG expression in skin and positively influence the skin homeostasis (Michalik and Wahli, 2007). To study the effects of the skin barrier function caused by a lack of FLG we established a FLG knock down skin model (Küchler et al., 2011; Vávrová et al., 2014).

We evaluated the effects of the PPAR agonist docosahexaenoic acid (DHA) in a FLG deficient skin model (FLG-) in terms of FLG expression via RT-PCR. Skin lipid organization was determined by ATR-FTIR and lipid composition was analyzed by HPTLC. Skin absorption studies were performed with the standard compounds testosterone and caffeine.

FLG expression increased significantly after DHA treatment. Histological examination revealed a thickening of the stratum corneum. DHA normalized the pathologically increased free fatty acid levels in FLG-. Furthermore, the skin lipid organization was significantly improved in FLG- constructs. Interestingly, skin absorption studies did not show an improvement of the skin barrier after DHA treatment.

Our data indicate that PPAR agonists do not exhibit their beneficial effects in inflammatory skin diseases by upregulating FLG, despite this leads to an improved lipid barrier, but probably mainly by anti-inflammatory effects.

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VI-1c-522 *

In vitro inflammatory 3D skin models of psoriasis and eczema as pre-clinical screening tools

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Psoriasis and eczema are prevalent diseases and pose a significant burden on patients. Ethical issues regarding experimental animal testing and interspecies differences, created a growing demand for *in vitro* models in which disease pathogenesis and therapeutics can be studied. We have developed 3D skin models using human primary keratinocytes to study inflammatory skin diseases and in which drug effects can be screened. Human skin equivalents (HSEs) exposed to psoriasis-associated cytokines express molecular markers specific for psoriasis (Tjabringa et al., 2008). Recently, we generated a sophisticated model by introducing T-cells in HSEs to study the crosstalk between T-cells and keratinocytes in a 3D microenvironment (van den Bogaard et al., 2014). T-cell-mediated cytokine secretion induced the expression of epidermal psoriasis markers. Drugs targeting the crosstalk between T-cells and keratinocytes reduced the inflammatory phenotype, suggesting that this model may be suitable in pre-clinical drug screening. To model eczema we added disease-associated cytokines IL-4 and IL-13 to HSEs to induce histological hallmarks of disease (Kamsteeg et al., 2011). HSEs from patient-derived keratinocytes facilitated the study in which we unraveled the molecular mechanism of an ancient dermatological therapy, which provided us with a new pharmacological target for drug development (van den Bogaard et al., 2013). Altogether, we are convinced that these skin disease models will aid in the identification of novel pathways for therapeutic strategies.

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VI-1c-625

Approaching an *in-vitro* model for atopic dermatitis

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The transferability of data generated in the animal appears to be limited (Hartung, 2013), human-based *in-vitro* disease models may allow for improvement in pre-clinical drug development. Reconstructed human epidermis/skin can make the step to skin disease models manageable which may improve the development of topical dermatics.

We develop a dual approach to the signs and symptoms of atopic dermatitis skin which includes the knock-down of the filaggrin gene (Mildner et al., 2011; Küchler et al., 2011) and inflammation induction of by cytokine exposure (Weindl et al., 2011). Filaggrin knock-down alters morphology, the order of stratum corneum lipids (Vávrová et al., 2014) and thus barrier function and response to irritants of reconstructed human skin (Küchler et al., 2011). Topical glucocorticoids improve the induced inflammation (Weindl et al., 2011). As nanoparticles can enhance the notoriously low skin penetration and even induce drug targeting to defined skin strata, the atopic dermatitis model is now used for the detailed investigation of topical drugs including tacrolimus which is beyond the preferable range of skin penetration because of a high molecular mass.

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VI-1c-782

An *in vitro* reconstructed psoriasis tissue model for evaluation of drug therapeutics

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Psoriasis is an inflammatory skin disease characterized by hyperproliferation and abnormal keratinocyte differentiation affecting 1-3% of the global population. Availability of an *in vitro* psoriatic tissue model will facilitate drug discovery. In the current study, normal human primary keratinocytes and psoriatic fibroblasts were harvested and cultured to

form a highly differentiated 3D tissue model. The tissue model was characterized for histological features, gene expression, and cytokine release patterns that are associated with psoriatic phenotype. Results showed that psoriatic fibroblasts can induce psoriatic phenotype *in vitro* with overexpression of HBD-2, psoriasin, elafin, and ENA-78, similar to the *in vivo* situation. Cytokine analysis showed increased release of IL-6 (7 fold), IL-8 (5.5 fold), and GRO-α (3.8 fold) compared to control tissues. Confocal microscopic evaluation revealed: 1) hyperproliferation of basal epithelial cells (Ki67 staining), 2) increased psoriasin, elafin, and CK16, and 3) reduced levels of filaggrin. Topical treatment of the tissue model with three over-the-counter psoriatic drugs decreased HBD-2, psoriasin, elafin, and ENA-78 gene expression. In conclusion, the *in vitro* psoriatic tissue model is anticipated to be a valuable tool to accelerate safety and efficacy studies of candidate therapeutics.

Session VI-1: *In vitro* disease models – Poster presentations

VI-1-009

Evaluation of *in vitro* inhibitory effect of Enoxacin on *Babesia* and *Theileria* parasites

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Enoxacin is a broad-spectrum 6-fluoronaphthyridinone antibacterial agent (fluoroquinolones) structurally related to nalidixic acid used mainly in the treatment of urinary tract infections and gonorrhea. Also it has been shown recently that it may have cancer inhibiting effect. The primary antibabesial effect of Enoxacin is due to inhibition of DNA gyrase subunit A, and DNA topoisomerase. In the present study, enoxacin was tested as a potent inhibitor against the *in vitro* growth of bovine and equine Piroplasms. The *in vitro* growth of five *Babesia* species that were tested was significantly inhibited ($P<0.05$) by micro molar concentrations of enoxacin (IC_{50} values = 13.5, 7.2, 7.5 and 24.2 μM for *Babesia bovis*, *Babesia bigemina*, *Babesia caballi*, and *Theileria equi*, respectively). Enoxacin IC_{50} values for *Babesia* and *Theileria* parasites were satisfactory as the drug is potent antibacterial drug with minimum side effects. Therefore, enoxacin might be used for treatment of Babesiosis and Theileriosis especially in case of mixed infections with bacterial diseases or in case of animal sensitivity against diminazin toxicity.

VI-1-025

Inter-laboratory validation of an innovative huFcERIa-RBL-2H3 degranulation assay for *in vitro* allergenicity assessment of whey hydrolysates

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Background: Cow's milk-derived whey hydrolysates are substitutes for allergic infants. Currently, huFcεRIα-RBL-2H3 cells, sensitized with serum IgE from cow's milk allergic patients, are used to assess *in vitro* residual allergenicity (van Esch et al., 2011). However, limited availability and inter-lot variation of sera impede standardization of safety testing using recombinant technology in degranulation assays.
Objective: An oligoclonal pool of chimeric (chu)IgE antibodies against bovine BLG was generated. Furthermore, an inter-laboratory ring trial was performed to investigate the reproducibility.
Methods: Six chimeric antibodies were generated comprising mouse variable domains and human constant IgE/K domains. These antibodies were tested for binding to the huFcεRIα-RBL-2H3 cells and their subsequent degranulation with whey, BLG or whey-based hydrolysates with different hydrolysation grades. The ring trial was performed with five different hydrolysates.
Results: Anti-BLG chuIgEs sensitized huFcεRIα-RBL-2H3 cells demonstrated degranulation upon cross-linking with whey, 18 kDa BLG, and 5-10 kDa hydrolysates, but not with a 3 kDa hydrolysate. The ring trial showed a good intra- and inter-correlation between four participating laboratories.
Conclusion: The huFcεRIα-RBL-2H3-assay using BLG-specific chuIgEs is very robust and reproducible. In addition, these *in vitro* data obtained matched previous outcomes of *in vivo* allergy models, underscoring the potential predictive value of this huFcεRIα-RBL-2H3-assay as alternative to animal studies.

Reference

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VI-1-192

An assessment of the cell stress response of lung epithelial cells exposed to cigarette smoke aqueous extracts

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The ability of cells to adapt and survive environmental and physiological stress relies on activation of cellular stress responses characterised by increased oxidative stress, inflammation and apoptosis. These processes occur in the development of many tobacco smoke related diseases. As one of a suite of *in vitro* models for a comparative assessment of tobacco related products, an *in vitro* lung model of the cell stress response was developed. Human bronchial epithelial (NCI-H292) cells were grown to confluence in 96-well cell culture plates and exposed to cigarette smoke aqueous extracts (CSEaq: 0-67%) from a reference (3R4F), a reduced toxicant prototype (RTP) and an equivalent commercial control cigarette for 4 hours. The intracellular ratio of reduced to oxidised glutathione, caspase 3/7 activity and secreted IL-1α, IL-6 and IL-8 were then measured as oxidative, apoptotic and inflammatory endpoints respectively. Exposure to 3R4F CSEaq induced a concentration dependent increase in all cell stress response endpoints. RTP CSEaq (67%), induced significantly lower oxidative, apoptotic and inflammatory (IL-6 only) responses compared to the commercial control. This model is useful in the comparative risk assessment of reduced toxicant prototype cigarettes and with development could also be applied to the assessment of electronic nicotine delivery systems.

VI-1-568

A model of neuronal hyperreactivity in passive sensitized human organotypic tissue

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Obstructive lung diseases are characterized by increased sensitivity of the airways (AHR) including peripheral neuronal hyperreactivity. Especially TRPV 1 positive non myelinated afferent C-fibers are believed to be involved in development of AHR. Local TRPV1 positive nerves are extensively co expressed with sensory neuropeptides. Local axon reflex response leads to the release of neuropeptides and provoke neurogenic inflammation including bronchoconstriction, vasodilation, inflammatory cell migration and mast cell degranulation. Using passive sensitized organotypic human precision cut lung slices (PCLS), it is possible to reflect partly asthmatic conditions. Capsaicin induced activation of the peripheral sensory neurons in passive sensitized PCLS leads to neurogenic inflammation including bronchoconstriction and mast cell degranulation. Bronchoconstriction was analysed by videomicroscopy using cross-sectioned airways in PCLS. Mast cell degranulation was determined by histamine ELISA and confocal microscopy. In contrast to *in vivo* asthma models in mouse, rat or guinea pig, *ex vivo* human PCLS predict human situation regarding peripheral nerve and neuropeptide composition, airway constriction in response to neuropeptides, local immune cell answer and airway microanatomy. Thus passive sensitized PCLS provide a suitable model to analyse features of obstructive lung diseases, like neuronal hyperreactivity, without the use of animal experiments.

VI-1-571 *

Human organotypic cancer model

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Multiple xenocraft mouse models have been generated to understand cancer, with the disadvantages of less predictive, expensive or technically complicated procedures. Here we present an innovative *ex vivo* organotypic tumor invasion model using living human precision-cut lung slices (PCLS) and cancer cells.

An AdGFP transduced human breast cancer cell line MDA MB 231 was added to human PCLS over a period of one week. Viability assays show intact human tissue during the infection with the cancer cells. Growth curves and Ki67 staining reflect proliferation of cancer cells over the observation period time in human PCLS. Immune response and neoangiogenesis were determined by the cytokine markers VEGF, IL 10, IL 1beta and GM CSF. The decrease of the proinflammatory cytokine IL 1beta was linked to the number of MDA MB 231 associated macrophages in human PCLS. The model mimics cancer cell proliferation in the microenvironment of human tissue without using artificial substances. It provides the possibility to gain insights into functional local immune responses with human physiology background. The model can be adjusted to other cancer targeted organs. In terms of the 3R concept, this alternative model does not require any animal experiments and takes advantage of human tissue.

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VI-1-578

Nucleus pulpous and annulus fibrosus cell isolation from human intervertebral disc

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Introduction: To study mechanisms of intervertebral disc (IVD) degeneration, *in vitro* organ culture systems with live disc cells are highly appealing. Most disc cells are isolated from animal tissue. We established a relatively quick and easy protocol for isolation of nucleus pulposus (NP) and annulus fibrosus (AF) cells out of the IVD fragments.

Materials and Methods: Human intervertebral disc fragments can be obtained following discectomies. In sterile conditions, disc fragments are collected. The tissue is cut, grinded and partially digested with trypsine. After centrifugation, sediment is harvested and cells seeded in suspension, supplemented with special media containing high nutrient level. Characterization was made and sub-isolation of nucleus NP and AF cells followed.

Results: In appropriate environment, isolated cells retained viabil-

ity and proliferated quickly. Both NP and AF cell cultures were stabile. Under standard culture conditions, cell proliferation and cluster formation was observed. Cell viability was 90%. The number of apoptotic cells and enucleated cells was positively correlated to cell seeding density.
Conclusions: The demonstrated isolation process is simple, quick and economical, allowing viable long-term organ culture. The availability of such system permits study of cell properties, biochemical aspects and therapeutic candidates for human discs in a well-controlled environment.

VI-1-730

Low concentrations of cigarette smoke extract act cytotoxic on human alveolar epithelial cells type II

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Alveolar epithelial cells type II (AECII) play an important role in normal pulmonary function, host defense, and immune response. The human tumor-cell line A549 is the most popular model of AECII.

In the presented study, the effects of cigarette smoke extract (CSE) on human primary AECII, A549, and the adenocarcinoma-cell line NCI-H1975 were investigated.

Tumor-free lung tissue from patients who underwent lobectomy due to cancer was used to isolate AECII. CSE was obtained using commercially available cigarettes by drawing smoke of one cigarette into 10 ml ddH_2O (=10% CSE). Cells were stimulated with increasing concentrations of CSE (0.1-5%) for 1 h. Cells were cultured for further 4 h. Cell viability was measured via MTT-assay.

Acute CSE-exposure with 0.5%CSE significantly induced cytotoxicity in AECII, which was not reversible by anti-inflammatory treatment. In H1975, 5%CSE were cytotoxic. No cytotoxicity was detectable in A549 cells.

Primary AECII display a model better suited to investigate cigarette-smoke-induced-inflammatory effects, than A549. Moreover, CSE-induced AECII damage is not reversible by short-term anti-inflammatory treatment.

VI-1-788

Chorioallantoic membranes as a living support for skin and human tumor cells xenografts

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Among the various alternative methods to animal models, we have developed grafting on the chick chorioallantoic membrane (CAM) in a shell-less model. This *ex-ovo* model includes many advantages such as the simplicity of *in vitro* cultures as well as the relevance of the *in vivo* models in addition to a low cost. Three axes were developed.

Pig full-thickness skin which closely resemble human skin, were grafted on the CAM to investigate percutaneous xenobiotic penetration for more than one week. This alternative had a main advantage over Franz cells which was limited at about a day of analysis. Transepidermal water loss remained unchanged during this period.

Human skin basocellular carcinoma can be grafted either as tumor fragments or as dissociated cells which modify vessel angiogenesis of CAM and induce vascularisation nearby, or within, the implants.

Human prostate tumor cells were also implanted on CAM. The dissociated tumor cells formed spheroids that stimulated neo-angiogenesis as soon as 48 h post-xenograft. Prior to implantation, the human tumor cells could be labelled with fluorescent dye in order to follow the tumor cells that have disseminated.

This model is a particular efficient system for studying long-term skin penetration or tumor development within an environment similar to natural conditions.

VI-1-840

Application of *in vitro* skin models for cosmetic product efficacy: UV protection, skin lightening, skin hydration and anti-aging

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In vitro skin equivalent models offer the capability to screen for safety and efficacy of raw cosmetic materials and formulations intended for topical application. Here we describe three commercially available skin tissue models, EpiDerm™, EpiDermFT™ and MelanoDerm™ that are amenable to multiple applications of interest related to cosmetic product development and efficacy testing. EpiDerm™, produced from normal human keratinocytes, is widely used for assessment of irritation, percutaneous absorption, cytotoxicity, cytokine release and has recently been utilized for evaluation of skin hydration. EpiDermFT™ is a full thickness *in vitro* skin equivalent produced from primary keratinocytes and primary fibroblasts containing a functional barrier and fully developed basement membrane. This model is well suited for evaluating cosmeceutical endpoints such as UV protection (e.g., CPD analysis) and skin aging biomarkers related to extracellular matrix remodeling. MelanoDerm™, a tissue containing primary keratinocytes and melanocytes, can be used to evaluate skin lightening following treatment with topically or systemically applied cosmetic ingredients allowing for measurement of macroscopic darkening and melanin production. Utilization of these tissue models for cosmetic product testing can be highly valuable in streamlining product development efforts and reducing the use of animals for testing purposes.

Session VI-2: Use of stem cells in screening

Co-chairs
Mario Beilmann, Boehringer-Ingelheim, Germany
Adrian Roth, Hoffmann-La Roche, Switzerland

Session VI-2: Oral presentations

VI-2-275

The DNT-EST: a predictive embryonic stem cell test for developmental neurotoxicity testing *in vitro*

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As the developing brain is exquisitely vulnerable to chemical disturbances, testing for developmental neurotoxicity of a substance is an important aspect of characterizing its tissue specific toxicity. Embryonic stem cells can be differentiated toward a neural phenotype, and this can be used as a model for early brain development. We developed a new *in vitro* assay using mouse embryonic stem cells (mESC) to predict adverse effects of chemicals and other compounds on neural development – the so-called DNT-EST (Visan et al., 2012; Hayess et al., 2013). After treatment of neurally differentiating stem cells for 48 h or 72 h at two key developmental stages, endpoints for neural differentiation, viability and proliferation were assessed. As a reference, we treated undifferentiated stem cells in parallel, also measuring viability and proliferation. Here, we show that chemical testing of a training set comprising nine substances allows the formulation of a mathematical prediction model that can discriminate positive from negative DNT compounds with an *in vivo – in vitro* concordance of 100%. Based on these results our current work aims to establish three-dimensional cortical tissues from embryonic stem cells to better model the complexity of the central nervous system for *in vitro* neurotoxicity assays.

References

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Hayess, K., Riebeling, C., Pirow, R. et al. (2013). *Toxicology 314*, 135-147.

VI-2-532

Human stem cell-derived cardiomyocytes in cardiac safety research

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Induced pluripotent stem cell-derived cardiomyocytes (hIPS-CMs) are increasingly used as a new source of cardiac cells for drug safety assessment. We investigated the effects of 20-60 reference compounds (mostly known to have cardiac effects) in 4-different types of stem cell-CMs, and using 5-different technologies, including two high content screens (HCS)-Ca^{2+} transient measurements, multielectrode Array (MEA), optical action potential, and xCELLigence. We also compared data of 20-reference compounds *in vitro*-isolated rabbit wedge model. Our data suggested that 1) using hiPS-CMs with HCS screen technologies (Ca^{2+} transients and Optical AP) could be suitable to detect drug-induced QT-prolongation, shortening, and increase in beat rate; 2) xCELLigence technology could be used to detect drug-induced chronic/delayed (days to weeks) cardiac effects *in vitro*; 3) *hES*-CMs and hIPS-CMs from different cell providers and different technologies could result in some different readouts. 4) The current HTS stem cell technologies do not differentiate causal mechanisms of certain different classes of drugs (e.g., Ca^{2+} channel blockers from IK_{ATP} channel openers), and additional lower throughput *in vitro* assays (ion channels) and *in vivo* assessments are still needed to clearly define MOA and ultimate risk position of new NME's.

VI-2-545

Defining normal developmental dynamics for human *in vitro* neuronal differentiation – applications for setting a baseline for adverse outcome assessments

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Increasing reliance on *in vitro* models for developmental toxicity demands careful characterization of these models. We have previously adapted a human neural progenitor cell (hNPC) model to assess developmental neurotoxicity. To assess relevance for *in vivo* development, we differentiated hNPCs up to 21 days and evaluated changes in pro-

tein expression by western blotting and immunofluorescence. Global gene expression was evaluated using Affymetrix Human gene 2.0 ST arrays. Under differentiating conditions, hNPCs increasingly express neuronal differentiation markers (including beta tubulin III, MAP2, and synaptophysin) and adopt morphological characteristics of mature neuronal networks. Pathway analysis reveals that GO terms enriched among genes decreased through time are largely associated with proliferation, and stem cell maintenance. GO terms enriched among genes with significantly increasing expression through time are dominated by key developmental processes, including neuronal differentiation, migration, and synaptogenesis. Enrichment of several GO terms associated with forebrain development indicates that our culture conditions promote differentiation towards a forebrain identity. We compared *in vitro* pathway dynamics with pathway dynamics apparent in publicly available data from developing human brain tissue. Key processes important for the identification of AOPs of proliferation, differentiation, and functional maturation matched *in vivo* patterns.

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VI-2-905

Stem cell derived tissues and the 3Rs: application in drug discovery and development

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The ability to make pluripotent stem cells from adult somatic cells has revolutionized cell biology, especially as access to differentiated tissues from ips cells has improved. The initial work has shown that ips cell derived tissues are more similar to intact tissue than cell lines or primary culture and rodents thus have begun to change the standard models to more human *in vitro* based models. In this talk, I will highlight the numerous examples where stem cell derived tissues have replaced, refined, and/or reduced the reliance on animal models in drug screening, toxicology, and product safety.

VI-2-906

Development of cardiovascular disease models in humans using iPS cells

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- Explanation of IPS cells and human embryonic stem cells.
- Generation of cardiomyocytes in a high throughput manner under defined conditions.
- How can we use human cardiomyocytes for screening.
- Latest developments on assays.
- Examples of drugs and cardiac toxicity using human stem cells.
- Personalized medicine: screening/testing of drugs in different groups of patients. Creating safer and more effective medicine (and reducing number of animals).

VI-2-907

Neural stem cells as screening tools for developmental neurotoxicity

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Cell-based assays that model key cellular events have been proposed for high throughput screening of chemicals for developmental neurotoxicity. While *in vitro* systems cannot fully replicate the complex temporospatial development of the brain, neuronal cultures can recapitulate neurodevelopmental processes such as cell proliferation, differentiation, growth, and synaptogenesis. Neuronal cell lines and rodent primary neurons have been used as *in vitro* model systems; however, cell lines do not always recapitulate the phenotype of a primary cell, and rodent-derived neurons may not capture early developmental processes or potential species-specific effects in human cells. Neural stem cells represent an alternative model system for studies of neurodevelopment *in vitro* and have several advantages including the property of self-renewal, the ability to generate the major cell types of the nervous system, and the availability of cells from multiple species including humans. We have used embryonic stem cell-derived neuroprogenitors, neuroprogenitors from fetal brain, and induced pluripotent stem cells in the course of evaluating cell-based assays for the neurodevelopmental processes of proliferation, differentiation, and neurite outgrowth. All of the models used were grown as adherent cells in 96-well plates allowing for endpoint quantitation using high content imaging. We observed differences in the sensitivity of cells to chemical effects on proliferation and neurite growth, with human cells being generally more sensitive compared to rodents.

This abstract does not necessarily reflect U.S. EPA policy.

Session VI-2: Poster presentations

VI-2-040

Genome-wide gene expression analysis by RNA-seq in murine embryonic stem cells

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Although it is not yet possible to replace *in vivo* testing completely, we can refine and reduce the number of animals used today. Here, we focus on a tiered approach combining genome-wide gene expression analysis by RNA-seq in murine embryonic stem cells (mESCs) and development of mathematical models. We firstly exposed model chemicals including Bis(2-ethylhexyl) phthalate and p-dichlorobenzene in 1 or 28 days (daily dose) to mESCs, followed by total RNA were isolated, and deep sequencing analysis were performed. Our results indicate that non-coding RNAs (ncRNAs) (Tani and Torimura, 2013) as well as mRNAs respond to the model chemicals. Next, for each chemical, we established no observed effect level (NOEL) values (mg/kg/day) for the effects involving several targets with respect to 28 days repeated dose toxicity studies, using existing oral experiment data on mice. Then, we performed statistical comparisons between our *in vitro* data and existing *in vivo* data. In addition, we discussed the results with reference to the Adverse Outcome Pathway (AOP) concept. We propose that the novel tiered approach will allow interim decisions to obviate further animal testing.

Reference

Tani, H. and Torimura, M. et al. (2013). *Biochem Biophys Res Commun 439*, 547–551.

VI-2-330

Establishing an *in vitro* screening test for developmental neurotoxicity

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According to US EPA OPPTS 870.6300 and OECD TG426, the chemicals evaluation of developmental neurotoxicity(DNT) is conducted with living animals, mostly rodents, which is time consuming, laboring and cannot satisfy with the requirement of testing so many chemicals. PSCs carry the characteristic of self-renew and differentiated into other cell types including neuron. Since the embryonic stem cell test(EST) may not fully suitable for DNT screening, it is necessary to develop a DNT screening method. Based on the mES, we tried to induce it to differentiate into neuron and glia cells by varies ways. After identification, 4 compounds were selected for test, "gold standard" (methyl mercury), the negative materials(Mannitol), generally cytotoxic compounds (5-FU) and pathway specific tool compounds (cyclopamine). Then the technologies based on electric cell-substrate impedance sensing(ECIS), high-content imagine analysis and flow cytometric cytotoxicity were used to observe and analysis the changes after exposure which including cell migration, neurite outgrowth, acethylcholine esterase and apoptosis Meanwhile the chemicals exposure to primary neuron and cell line also compared. With preliminary validation, the alternative approaches can be used to screen chemicals and accelerate collection of evidence on higher priority chemicals.

VI-2-507 *

Cardiomyocytes from pluripotent stem cells – a promising alternative in drug assay

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Organ specific stem cells of animal origin have potential application in dug assays and regenerative medicine. They are harvested from spent animal, cultured *in vitro*. Pluripotent stem cells used in the present study were derived from goat uteri containing undifferentiated fetus. The fetus were collected from slaughter house, sterilized, centrifuged and washed with Dubecco's PBS. The fluid containing the desired cells was washed in CR11 media and incubated at 38.5°C, 5% CO_2 and 90% RH in CO_2 incubator for a period of 7 days. Developed stem cell clones were mechanically disrupted and re-cultured in CR11 media with Lipopolysaccharide at the concentration of 2-8 μg/ml. The re-culturing was continued till 3rd passage. After that Lukemia Inhibitory Factor (LIF- a media ingredient in CR11) was removed from the media and cells were allowed to get differentiated. Clones of beating cardiomyocytes like cells were observed in the culture plates on 28th day of the passaging. Transcript level of cardiac specific gene Nkx 2.5 was studied on these cells. Cardiomyocytes developed in the present study could be potential alternative to the use of lab animals for cardiac drug assays and toxicological studies. Further studies on these cells are progressing.

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VI-2-664

Profiling of drugs and environmental chemicals for functional impairment of neural crest migration in a novel stem cell-based test battery

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The EU-FP7 project "embryonic stem cell-based novel alternative tests" (ESNATS) aimed at establishing a test battery framework for developmental toxicity. Here, we present the overall test battery concept, suitable for a wide range of *in vitro* toxicity assays (Zimmer et al., 2014). A screen library of 28 compounds (pharmaceuticals and environmental toxicants) was selected and characterized for cytotoxicity as well as for clinical and toxicological data. To evaluate the feasibility of the test framework, the "migration inhibition of neural crest cells" (MINC) assay was chosen (Zimmer et al., 2012). Screening at the highest non-cytotoxic concentration resulted in 11 hits. To further understand the mechanisms underlying the observed inhibition, a microarray analysis was performed for some selected hits and the differentially expressed genes were analyzed by GO term enrichment analysis. This study confirmed the potential use of the MINC assay for the prioritization of substances belonging to different chemical classes. In conclusion, this feasibility study points out important design principles of a test battery for identification of reproductive toxicants. Our approach shows a potential strategy for the combination of assays available in different laboratories to provide more information both on the assays themselves and also on the compounds included in the screens.

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Session VI-3: Human biomarkers

Co-chairs
Barry Hardy, Douglas Connect, Switzerland
Andre Schrattenholz, ProteoSys, Germany

Session VI-3: Oral presentations

VI-3-807

The automated FADU assay: an alternative method for monitoring DNA repair in human studies

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Our genome is constantly challenged by exogenous and endogenous damaging agents; therefore cells have developed DNA repair mechanisms to ensure genomic integrity. However, there are many influences that can compromise DNA repair resulting in DNA damage accumulation, which might lead to diseases, cancer or accelerated ageing. Therefore, establishment of alternative methods for monitoring DNA repair in human cells is of utmost importance for a wide variety of scientific fields. The automated Fluorometric Analysis of Alkaline DNA Unwinding (FADU) assay provides a reliable, convenient and objective method to quantify DNA breakage *in vivo*. In the context of a scientific multi-center cooperation, we investigated associations of subjective vitality with DNA damage as well as age, gender and genetic and environmental influence on DNA strand breaks. In another cooperative study we assessed DNA strand breaks and the immediate repair phase in blood cells from individuals with posttraumatic stress disorder before and after psychotherapy. Our results revealed that exposure to traumatic life events is associated with higher levels of DNA damage and we presented a proof-of-principle for the reversibility of DNA strand breakage after successful psychotherapy. In our study, gender and genetic background had no significant effect on DNA strand break repair.

VI-3-918

Use of alternative evidence in replacement research and safety assessment supported by OpenTox and ToxBank

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Data on SEURAT-1 Gold Compounds (SGCs) were obtained from the literature and organised and made available through the ToxBank wiki and data warehouse. The data integration included transcriptomics data from TG-Gates, assay data from PubChem, toxicokinetics data and parameters from the literature, and *in vivo* data resources. Interoperability between ToxBank components and other resources was based on service implementation of OpenTox standards. Analysis methods for Read Across, enriched meta analysis of multiple omics and functional data, background knowledge from GO ontologies and Kegg pathways, and pathway visualisation were developed and ap-

plied to the SGCs. We present an analysis case study for Doxorubicin based on publically available data examining the variation of pathway interactions as a function of dose and time. Using SGC examples, we discuss the differing information requirements and solutions for computational and expert-based components to Read Across and Weight of Evidence for the following contexts with consideration of the value and current limitations in Alternative-based evidence:
a) Hypothesis-driven mechanistic-based research
b) Predictive goal of an integrated testing strategy
c) Incorporation of evidence in safety assessment decision making.
The role of such methods in providing evidence and guidance supporting biomarker development will be discussed.

VI-3-919

Human biomarkers of disease, from discovery to validation and test development

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There are quite diverse phenotypic effects resulting from toxic interventions or pathological conditions, which are accessible for measurements with modern OMICS-technologies. They include a variety of nucleic acid species (RNA's and DNA) and mass spectrometry (metabolites, proteins, lipids, sugars). Toxic effects depend on individual genetic, epigenetic and environmental predispositions and conditions.

Epigenetic and environmental effects are to a large extent present on the proteomic level. Proteins also show the most immediate molecular effects in time-resolved experiments. The kinetics of cellular responses requires special attention with regard to data types and formats, data models and ontologies and interface solutions for integration will be discussed.

Working with human *in vitro* models requires the establishment of kinetically controlled SOP's for sample generation, processing and storage, meta data tracking frameworks (like, e.g., ISATAB). The aim is to define sets of biomarkers exactly describing molecular initiating events and the downstream key events which eventually cause adverse outcomes.

In terms of molecular biomarkers, in a first stage a thorough statistical analysis of raw data will reveal consistent quantitative data signatures plausible across samples and conditions. This is pivotal to exclude effects of contamination and unrelated biological activity. A clear definition of biological and data acquisition criteria will result in the selection of validated data sets.

In a second stage the whole validated data set or selected subgroups of biomarker candidates will be searched according to biological criteria. In the toxicological projects investigated so far (SEURAT-1, Reprotect) one of the hall marks was oxidative stress, contributing to a cascade of specific posttranslational modifications (oxidative, glycation), some of them directly accessible by mass spectrometry (e.g., N-formyl-kynurenin modification).

Obviously the number of pathways is relatively limited and these pathways are organized in flexible and redundant feed-back systems. Certain layers of omics analyses reflect better or worse the kinetics of reactions in these pathways of stress and escape responses.

Predictive modelling will require adequate incorporation of kinetic information and treatment of feed-back and feed-forward mechanisms.

VI-3-920

The development, use and interpretation of *in silico* models to support the ICH M7 guideline

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The International Committee on Harmonisation (ICH) has recently issued a draft guidance (currently out for public comment – termed step 2) that covers the qualification of mutagenic impurities (ICH M7). In the absence of carcinogenicity or bacterial mutagenesis data for actual or potential impurities, an *in silico* structure-activity analysis can be performed to help understand whether a substance can be classified as having no mutagenic concern. To perform the computational structure-activity analysis, the guideline states that two complementary *in silico* methodologies should be used in the assessment. One should be expert rule-based and the second should utilize a statistical-based methodology. This presentation will outline how rule-based expert alerts systems as well as statistical-based QSAR models are developed and used to generate predictions to support the assessment of impurities as part of the ICH M7 guidance. The presentation discusses how the results can be combined to generate a consensus prediction as well as how these results can be used to support an expert opinion.

Session VI-3: Poster presentations

VI-3-005

Funding innovation to foster decreases in compound attrition and the 3Rs: a new process

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Despite the considerable advances in science and technology in the biological space in the last two decades, research in pharmaceutical industry is still affected by a high rate of attrition of compounds during the drug development process. Similarly, our reliance on laboratory animals is high, despite the strong commitment of the application of the 3Rs principles in R&D processes. Attrition and 3Rs are inherently linked: in general, changes that would reduce compound attrition have also an impact on 3Rs.

A new process for identification and funding technological opportunities with an impact on attrition and the 3Rs has been set up in GSK. This presentation is aimed to illustrate the GSK model.

VI-3-411

Proton magnetic resonance spectroscopy and recursive partitioning analysis of brain metabolism data from an Alzheimer's disease cohort offers a replacement alternative for animal studies on disease prediction

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Fundamental research into Alzheimer's disease (AD) relies on animal models for the investigation of disease mechanism and new therapeutics. Transgenic rodent strains are available (Kitazawa et al., 2012) with mouse models used for brain chemistry to memory loss studies (Murakami et al., 2011; Cassano et al., 2012). Also, micro-imaging methods have been used to study a transgenic rat AD model (Teng et al., 2011). A combination of a clinically and genetically well-defined human cohort, proton magnetic resonance spectroscopy (^{1}H-MRS) and pattern recognition analysis of cohort data (Londono et al., 2013) offers a replacement alternative to animal models that predict AD onset features. Seventy-five participants from a multigenerational AD pedigree were divided into; (1) carriers of the fully penetrant mutation in Presenillin-1 (n=44), and (2) non-carriers (n=31); seventeen carriers had mild cognitive impairment (MCI) or early-stage AD. ^{1}H-MRS was conducted on each participant, with brain metabolite data (e.g., choline/creatine ratio) for white and grey matter regions of the posterior cingulate gyrus and precuneus included with demographic data, prior to recursive partitioning analyses. Metabolite ratio profiles successfully discriminated carriers and asymptomatic carriers. Brain metabolites measured by ^{1}H-MRS are optimally sensitive and specific non-invasive biomarkers of subclinical emergence of AD caused by the PSEN1 mutation, achieved without pre-clinical animal experiments.

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VI-3-823

From transient transcriptome responses to disturbed neurodevelopment: divergent response patterns and epigenetic modifications triggered by the same drug

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Literature often assumes that toxicogenomics data reflect the immediate mode of action (MoA) of drugs. Alternatively, transcriptome changes could describe altered differentiation states as indirect consequence of drug exposure. We addressed this question in differentiating human embryonic stem cells exposed to TSA. Histone acetylation (primary MoA) increased quickly and returned to baseline after 48 h. Histone H3 lysine methylation of the neurodevelopmental regulators PAX6 and OTX2 was affected and remained persistent. These alterations correlated with neurodevelopmental defects and with changes in PAX6 expression, even with a drug washout after 3 days.

We hypothesised that drug exposures altering only acetylation lead to reversible transcriptome changes (indicating MoA) and altered methylation leads to irreversible developmental disturbances. Data from pulse-chase experiments corroborated this assumption. Short drug treatment triggered reversible transcriptome changes; longer exposure disrupted neurodevelopment. The disturbed differentiation was reflected by an altered transcriptome pattern. The changes were similar after a 4 days washout. We conclude that transcriptome data after prolonged treatment of differentiating cells mainly reflects the altered developmental stage of the model system and not the drug MoA. We suggest that brief exposures, followed by immediate analysis, are more suitable to study immediate drug responses and MoA.

VI-3-825

Waves of gene expression as basis to define windows of sensitivity for developmental neurotoxicity

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In the field of neurodevelopmental toxicity there is an urgent need for appropriate *in vitro* tests. We established a human embryonic stem cell based test system. However, the major challenge of such a system is that gene expression patterns change already in untreated control conditions. Therefore, we hypothesize that not only concentration and duration of treatment matter but also the time window. We used microarray analysis to get (i) a closer insight into the underlying processes of neurodevelopment and (ii) to investigate drug effects for different

treatment scenarios. Gene expression analysis at different time points of undisturbed differentiation showed that gene regulation proceeds in a wave-like pattern. Using the HDAC inhibitors VPA and TSA as well characterized DNT compounds, we treated the cells for 6h/4d/6d. We found that developmentally regulated genes and drug regulated genes overlapped up to 90% at late time points but showed only a small overlap at early time points. This may allow differentiation between cell biological and developmental toxic effects. For phenotypic anchoring, gene expression changes were correlated with the capacity of treated cells to still form neural tube like rosettes.

Session VI-4a: Absorption, distribution, metabolism and excretion (ADME)

Co-chairs
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Session VI-4a: Oral presentations

VI-4a-424

SPECT-CT imaging to study the pharmacokinetics of radiolabelled antisense oligonucleotides (AONs) *in vivo*

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Aim: For drug development, determining the biodistribution of compounds is extremely important. SPECT-CT imaging is a non-invasive technique, allowing repeated measurement of biodistribution *in vivo*. Currently, availability of dedicated non-invasive technologies to evaluate pharmacokinetics of DNA-/RNA-based therapeutics, such as AONs, is still limited. We aimed to set-up and validate novel minimally-invasive methods to trace AONs *in vivo*.
Methods: An AON developed for Duchenne Muscular Dystrophy (DMD) treatment was radiolabeled with either ^{123}I or ^{111}In. The pharmacokinetics of [^{123}I]-AON or [^{111}In]-AON after subcutaneous administration (100 mg/kg) to Mdx mice, a model for DMD, were assessed with SPECT-CT imaging. Subsequently, tissues were collected, radioactivity counted and AONs quantified by an ELISA-based method.
Results: SPECT-CT imaging showed that both [^{123}I]-AON and [^{111}In]-AON could be used to determine in situ tissue levels of the AON up to 48 hours after administration. Scan data matched well with results from the invasive biodistribution.
Conclusions: Quantitative SPECT-CT imaging with radiolabelled AONs provides a powerful approach to non-invasively assess AON pharmacokinetics. Radiolabelling with ^{111}In appears preferable for oligonucleotides. Imaging can significantly improve translation to humans, and reduce the number of animals used in preclinical drug development.

VI-4a-539

Living cell as a tool in *in vitro* toxicity testing platforms

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Toxicokinetic behaviour of most small organic xenobiotic chemicals is mostly determined by xenobiotic-metabolizing enzymes. Thus, in toxicity testing platforms, be it non-cellular or cellular system, it is important to ensure that xenobiotic metabolism is functional or incorporated and at levels analogous to those *in vivo* or at least useful for quantitative *in vitro-in vivo* extrapolation (QIVIVE). Although non-cellular testing systems with metabolic competence are widely used in *in vitro* toxicity testing, living human cells in 2D and 3D configurations are the most versatile tool for *in vitro* toxicity tests. However, cellular systems should be developed in a more detailed way than thus far, taking into account the early characterization of metabolism and other important kinetic processes, because the thorough characterization is a necessary for the validation and use of cellular systems for toxicological studies. In addition, because metabolism is not the only important factor to be incorporated into toxicokinetic predictions, a more comprehensive "systems toxicology" approach should be incorporated into any QIVIVE exercise.

VI-4a-798

Gaining insight into xenobiotic biotransformation: the CYP induction *in vitro* method

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Cytochrome P450s (CYP) are Phase I biotransformation enzymes that are frequently responsible for transforming both endogenous and exogenous compounds. EURL ECVAM used induction of CYP enzymes produced by exposure to xenobiotics to evaluate the metabolic competence of two human-derived *in vitro* hepatic test systems. The multi-study validation trial assessed the reliability and relevance of CYP induction methods to evaluate the functional induction of four CYP enzymes (CYP1A2, CYP2B6, CYP2C9 and CYP3A4) in two *in vitro* test systems: cryopreserved human HepaRG® cells (Andersson et al., 2012) and cryopreserved human primary hepatocytes (Richert et al., 2010; Alexandre et al., 2012; Yajima et al., 2014). The predictive capacity was assessed by testing 10 (HepaRG®) or 12 (cryopreserved primary hepatocytes) compounds and comparing the results to human *in vivo* CYP induction reference data. Since the CYP induction method is based on xenobiotic-nuclear receptor binding, dimerization, activation of DNA binding domain and enhanced transcription of the target gene, we predict that any class of compounds that can interact with such receptors can be used in the *in vitro* human CYP induction methods. Based on this project, OECD member countries accepted the development of a new test guideline as an addition to the OECD work programme.

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VI-4a-914

In vitro to *in vivo* extrapolation and reverse dosimetry

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The field of toxicology is currently undergoing a global paradigm shift to use of *in vitro* approaches for assessing the risks of chemicals and drugs, yielding results more rapidly and more mechanistically based than current approaches relying primarily on *in vivo* testing. However, reliance on *in vitro* data entails a number of new challenges associated with translating the *in vitro* results to corresponding *in vivo* exposures. A combination of *in silico*- and *in vitro* parameter estimation, together with pharmacokinetic modeling, can be used to predict the *in vivo* exposure conditions that would produce chemical concentrations in the target tissue equivalent to the concentrations at which effects were observed with *in vitro* assays of tissue/organ toxicity. This presentation will describe the various elements of IVIVE and highlight key aspects of the process including: (1) characterization of free concentration, metabolism, and cellular uptake; (2) conversion of *in vitro* effect concentrations to equivalent human exposures, and (3) potential complications associated with metabolite toxicity. Two examples of PBPK-based IVIVE will be described: a simple approach using whole hepatocyte clearance and plasma binding that is suitable for a high-throughput environment, and a more complicated approach for a case of metabolite toxicity. Recent efforts to improve the *in vivo* relevance of *in vitro* kinetic data will also be described.

VI-4a-916

The added value of physiologically-based pharmacokinetic in modelling the target tissue exposure for PKPD analysis

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Although the new generation of physiological-based pharmacokinetic (PBPK) models is now embraced by the pharmaceutical industry as well as regulatory organisations (Ho et al., 2014), the advantages of these models in estimating the local concentrations in various tissues over time has not reached its full potential. The drug safety or pharmacological effect is usually linked to the local exposure in the tissue. However, this is not directly measured. It is well known that variation in the local concentrations due to the effect of variable transport can blur the distinction between PK and PD variability. Various genetic variations in the functionality of uptake or efflux transporters have recently been discovered at blood brain barrier, hepatocytes, or proximal kidney tubule. PBPK models with "permeability-limited" organ models can account for transport-mediated uptake or efflux and assess the local concentrations (Neuhoff et al., in press; Hsu et al., 2014; Jamei et al., 2013).

Recently, Rose et al. (2014) reported the application of a PBPK model to predict the pharmacodynamics of rosuvastatin patients with impaired *OATP1B1*-related activity. Accordingly, there is a compelling argument for using PBPK in projecting PKPD when drugs are subject to polymorphic transporter activity. This approach can provide information on sub-groups which are rarely studied in clinic.

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Session VI-4b: Absorption, distribution, metabolism and excretion (ADME)

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Session VI-4b: Oral presentations

VI-4b-313

Organ-like three dimensional test systems

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Products must be tested for their quality, safety, and efficacy. 2D models have been widely used to analyze physiological effects. However, conclusions are limited and less obvious compared to the *in-vivo* situation. 3D cell cultures possess many features that mimic the *in-vivo* microenvironment which cannot be detected in the traditional 2D cell cultures. Therefore, we developed alternative human test systems that reflect the complex characteristics of the body. We work with three different skin models (Mertsching et al., 2008): an epidermal model, a full skin model and a vascularized skin model, which can be extended by other cells (melanocytes, tumor cells). The models are suitable for measuring penetration, distribution, and metabolization of test substances. Additionally, effects concerning the proliferation, differentiation and cell death can be examined. Our group has succeeded in building up both a trachea (Macchiarini et al., 2004) and an intestine (Pusch et al., 2011) test system based on primary cells and a matrix with a blood vessel system (BioVaSc®; Stratmann et al., 2014). We also work with different tumor model systems (Schanz et al., 2010) (intestine, breast, lung, Neurofibroma, leukemia) based on the BioVaSc®. To ensure the functionality of these cells *in vitro*, models are cultured in specific bioreactor systems with conditions similar to the natural microenvironment.

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VI-4b-672

Development of standards for characterising and describing *in-vitro* human hepatic metabolic clearance methods

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Hepatic metabolic clearance plays a key role in the transformation and the elimination of chemicals from the human body. In recent times various *in vitro* methods for human hepatic metabolic clearance/stability have been developed, employing different biological systems, monitoring either test item depletion or metabolite formation and including various test system configurations. This heterogeneity in *in vitro* clearance methods results in heterogeneity in *in vitro* clearance data. EURL ECVAM, acknowledging the importance of ADME information in the regulatory safety assessment of chemicals, is taking the initiative to develop harmonized standards for *in vitro* human hepatic clearance methods. These standards will be based on the results of (1) an evidence based literature research, (2) a test submission e-survey tool and (3) an expert workshop. The new standards aim to challenge the *in vitro* methods in relation to defined applications, to guide the end-user in using a harmonised reporting format and in providing estimations on the uncertainty of generated parameters. In this contribution, these emerging standards will be presented, along with the detailed workflow used to generate them.

VI-4b-711

New approach to predict human oral absorption using porcine intestinal tissue, abundance data and biorelevant matrices

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The predictive value of currently used models for oral bioavailability in humans is often insufficient, especially when food-drug interactions are evaluated. We aimed to design a new approach for optimal prediction of oral absorption. First, three different *in vitro* permeability models (Caco-2 cells, porcine intestinal tissue from re-used animals mounted in InTESTine™, and human intestinal tissue mounted in Ussing system) were characterized with regards to apparent permeability (Papp), absolute expression levels of transporters and metabolic enzymes. Papp values determined using the porcine InTESTine™ system were in the same range as using human intestinal tissue, whereas data obtained with Caco-2 cells showed major differences. These differences could partly be explained by different abundance of transporters and metabolic enzymes in comparison with human intestinal tissue. Moreover, we showed a unique combination of porcine InTESTine™ system with luminal samples collected from a gastrointestinal model (TIM) studying the permeability of compounds in the presence of undiluted biorelevant samples without loss of integrity. The results show that porcine intestinal tissue mounted in InTESTine™ system provides a good alternative for human intestinal tissue, and together with abundance data, biorelevant matrices and PBPK offers a unique combination for optimal prediction of oral bioavailability in humans.

VI-4b-836

Prediction of the brain distribution of drugs in human using *in vitro* human blood-brain barrier model derived from stem cells

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Blood-Brain Barrier (BBB) *in vitro* models can provide a valuable tool for studying mechanistic aspects related to the transport of drugs at the brain, as well as biological and pathological processes related to the BBB (Cecchelli et al., 2007). Although *in vitro* models were established from various species (rat, mouse, bovine, etc.), the development of a human BBB model is very important in order to account differences between animals and Human (Syvänen et al., 2013).

Here, we report a method to generate a stable and reproducible human *in vitro* BBB model using cord blood stem cells (Cecchelli et al., accepted) perfectly adapted for drug discovery and development (DDD). The use of this model according to new methodology developed by our lab (Culot et al., 2013) demonstrated for the first time a good correlation, between the human *in vitro* predicted unbound brain-to-plasma drug concentration ratio and the *in vivo* brain-to-cerebrospinal fluid unbound drug concentration ratio reported in humans.

These results support human relevance of data generated *in vitro* by our new human BBB model and confirm interest of *in vitro* human model to limit attrition at late stage in DDD for Human due to species specificity.

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VI-4b-891

In-vitro Buccal delivery and release of caffeine from Guarana extract

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Guarana is a climbing plant in the maple family, Sapindaceae, native to the Amazon basin and especially common in Brazil. As a dietary supplement, guarana is an effective stimulant (Johannes, L., 2010) its seeds contain about twice the concentration of caffeine found in coffee beans (Jump up Bempong et al., 1993). The aim of the study was to compare the transbuccal absorption of caffeine from guarana extract and pure caffeine in PBS solution and artificial saliva.

For this purpose, Animals obtained from slaughterhouse were used. Buccal mucosa was surgically removed from the porcine oral cavity, the connective tissue was then carefully removed and the buccal mucosal membrane isolated. The membranes were then mounted in Franz diffusion cells for the *in vitro* permeation experiments. In the literature different compositions of artificial saliva was found. For the purpose of this study modified Fusayama saliva was used.

The contained Caffeine in Guarana (50 mg/ml; containing the equivalent of 5,15 mg/ml caffeine) was found to permeate in the same way in artificial saliva and PBS. No difference could be show in comparison to pure caffeine contained in PBS or artificial saliva. It can be stated that artificial saliva has no effect on the permeation of caffeine.

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Session VI-4: Poster presentations

VI-4-213

Porcine buccal mucosa as an *in vitro* route model for systemic drug delivery evaluation

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Purpose: Determination of drug permeability across the buccal mucosa barrier generally speeds up the drug development process, and models are needed for this purpose (Nielsen, 2002).The aim of the present study was to standardize the porcine buccal mucosa as *in vitro* model (Mahalingam, 2007) for the examination of drug transport.
Methods: For this purpose, mucosal membrane, isolated from Animals obtained from slaughterhouse were used. The membranes were then mounted in Franz diffusion cells for the *in vitro* permeation experiments. The permeation of caffeine was evaluated on fresh, -4°C stored, and frozen (-20°C and -80°C) tissues. For the freezing conditions, glucose and glycerol were evaluated as cryopreservatives. ^{14}C-Mannitol was used as quality control.
Results: On fresh tissue the permeability coefficient (Papp) for caffeine showed reproducibility between the day 1 and the day 2. The very low Papp obtained for ^{14}C-Mannitol confirmed the tissue integrity. There were no differences observed for caffeine cumulative transport, between fresh and -4°C stored tissue. By using -20°C frozen tissue, the cumulative transport was the same for each cryopreservative used.
Conclusion: Based on these results it could be stated that frozen or fresh the porcine buccal mucosa can be used as model for evaluation of drug transport (Hoogstraate, 1996).

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VI-4-277

Characterization of monoamine oxidases, steroid-5α-reductases, sulfotransferases and glutathione S-transferases in reconstructed skin tissues and human skin *ex vivo*

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The use of reconstructed human skin (RHS) in preclinical development of topical dermatics and transdermal therapeutic systems requires detailed knowledge on the biotransformation capacity of the constructs. We investigated monoamine oxidases (MAO), steroid-5α-reductases (SRD5A) as well as sulfotransferases (SULT) and glutathione S-transferases (GST) in excised human skin, RHS (Phenion FT, Epiderm-FT) and in normal keratinocytes, HaCaT cells and fibroblasts.

Gene expression levels were determined by quantitative RT-PCR and MAO protein expression by Western blot analysis. GST activity was evaluated using 1-chloro-2,4-dinitrobenzene as a substrate.

MAO A, SRD5A type 1 and 3 were expressed in all matrices, whereas MAO B and SRD5A type 2 levels were higher in dermis than epidermis and hardly detectable in undifferentiated keratinocytes. In human skin and reconstructed tissues strong constitutive expression of SULT2B1b, SULT1E1, SULT1A1, GSTP1 and GSTT1 was found. GST activity was slightly higher in RHS compared to human skin.

Reconstructed tissues and human skin share a similar gene expression profile of the tested phase I and phase II enzymes and GST activity. Thus, RHS appear to be suitable for preclinical testing and toxicology studies with respect to biotransformation related processes.

VI-4-390

Influence of receptor fluid on dermal absorption of caffeine

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Dermal absorption describes the transport of chemicals from the surface of the skin into the skin and into the systemic circulation. An *in vitro* method, based on Franz cells, using preferably human skin is widely used for the investigation of penetration properties of substances. To ensure a correct interpretation of results it is important to investigate factors influencing this assay.

In the current study the influence of different receptor fluids on the penetration properties of caffeine was investigated. The study was performed in a dynamic flow through system using human skin. Caffeine was applied at the start of the study and receptor fluid was collected at various time points. At the end of the study all matrices (skin, strips, skin wash and receptor fluid) were analysed for the content of caffeine. The receptor fluids were water with 50% Ethanol and DPBS supplemented with BSA (5%), DMSO (5%) or SDS (5%).

The results confirmed, that the receptor fluid used for dermal absorption studies *in vitro* has an influence on the penetration properties. The total absorption of Caffeine varied depending on the receptor fluid composition between 45% (DMSO and BSA) and 15% (50% Ethanol and SDS).

VI-4-479

Microdosing of new biological entities (NBEs) in healthy volunteers: a safe and fast tool to predict clinical pharmacokinetics

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Aim: Microdosing, combined with Accelerator Mass Spectrometry (AMS), provides a safe approach to obtain first-in-human pharmacokinetic data, after very limited safety testing in animals. We investigated the microdosing approach to determine pharmacokinetics and safety of NBEs, using recombinant human Placental Alkaline Phosphatase (hRESCAP, a therapeutic protein under investigation for clinical application) as a model compound.
Methods: hRESCAP and [^{14}C]-hRESCAP were successfully, GMP-compliant, manufactured and characterized with *in vitro* methods. A microdose (53 µg) of [^{14}C]-hRESCAP was i.v. administered to healthy volunteers, and safety and pharmacokinetics were assessed. Subsequently, increasing doses (414, 1240, and 5300 µg hRESCAP; including 53 µg [^{14}C]-hRESCAP), were administered. [^{14}C]-hRESCAP plasma levels were determined by AMS, total hRESCAP by an enzymatic assay.
Results: Single doses of 53- 5300 µg hRESCAP, including 53 µg [^{14}C]-hRESCAP, were well tolerated. Pharmacokinetic analysis indicated dose-linearity from microdose to therapeutic doses.
Conclusions: A microdose of [^{14}C]-hRESCAP successfully predicted favorable plasma residence time of hRESCAP at therapeutic doses, and was a safe starting dose for a first-in-human study. Microdosing provides a safe and fast clinical development route for NBEs like hRESCAP, and can significantly reduce the number of animal studies.

VI-4-528

A comparison of xenobiotic metabolism enzyme profiles in human skin and *in vitro* skin models

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Cosmetics Europe funded and led two projects to characterise the metabolic capacity of human native skin and to compare it with a number of 2D and 3D *in vitro* skin models currently used in toxicity assays (Hewitt et al., 2013).

Phase 1 enzyme expression and activities were low in both native skin and the *in vitro* models. Some xenobiotic metabolising enzymes (XMEs) in whole skin were not detectable in *in vitro* models (e.g., dermal XMEs) and *vice versa* (e.g., AKR 1B), and some major hepatic XMEs, such as cytochrome P450s, were present only at very low levels or absent in native skin and *in vitro* skin models. By contrast, activities of phase 2 enzymes were all readily measurable in native skin and *in vitro* skin models at levels similar to those measured in the liver. Studies on the effects of genotoxins on EpiDerm™ models demonstrated that a test chemical itself can alter the XME levels during the course of the assay, as demonstrated by the induction of at least the CYP1 family by benzo[a]pyrene.

In conclusion, this comprehensive profile of XMEs in native skin and alternative *in vitro* dermal models has enabled a better understanding of their contribution to toxicity endpoints.

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Session VI-5: Epithelial biobarriers

Co-chairs
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Session VI-5: Oral presentations

VI-5-341

Development of human airway tissue-based assay for respiratory absorption giving input parameters for PBTK modeling

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Airway epithelium is a prime entry portal of xenobiotics into the body. Knowledge of toxicokinetics is needed to estimate the possible range of target doses at the cell or tissue level that can be expected from realistic human exposure scenarios to inhaled compounds (Bur and Lehr, 2008). Our study evaluated the permeability property of an *in vitro* cell model of the human airway epithelium (MucilAir™) (Reus et al., 2014). The absorption of test items was assessed after apical or basolateral exposures, and the permeability rate (Papp) of the chemicals across airway epithelium was measured. A standard operating procedure was developed and its transferability and reproducibility was evaluated using 6 chemicals (propranolol, atenolol, nicotine, cadmium-chloride, cobalt-chloride and ammonium-hexachloroplatinate) in two independent laboratories. A panel of 30 compounds were further tested to evaluate the ability of the assay to rank relative permeability. A comparative permeability study between nasal and bronchial epithelium has been performed. The results showed generally: (i) a higher permeability of the airway epithelium for organic compared to inorganic compounds and (ii) a low transporter-mediated efflux involved in the permeability. The study indicates that this MucilAir™-based assay represents a promising

tool to evaluate respiratory absorption giving input parameters for PBTK modelling (Bessems et al., 2014).

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VI-5-632

Design and characterization of *in vitro* models for human skin wounds

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Even though rodent skin is more permeable and excised human skin is considered as gold standard for drug absorption testing, rodent wound models are frequently used (Scott et al., 1986; Ansell et al., 2012). To address this issue and provide an alternative to animal testing, this study presents a reproducible *in vitro* skin wound model based on excised human skin. Three-dimensional skin wounds with linear, rectangular wound geometry and defined tilted wound edges were created (Rizzo et al., 2012; Planz et al, in reply). Further, for non-invasive wound characterization, optical profilometry and Raman spectroscopy were introduced realizing three-dimensional analysis of wound geometry and healing without tissue destruction (Kann and Windbergs, 2013). Virtual sectioning by these techniques provides a valuable alternative to invasive tissue histology. In direct comparison to established skin punch biopsies exhibiting variable wound size depending on the sectional plane, the linear shape of the novel wound model overcomes this problem, as visualized by profilometry scans and histological sections (Planz et al., in reply). In addition, the novel model provides a more realistic simulation of the human *in vivo* pathophysiology exhibiting tilted wound edges completely surrounded by intact tissue. The study presents a versatile *in vitro* model simulating human skin wounds which is reproducible as well as easily applicable and meets all requirements for valid testing of novel therapeutic systems at the site of action.

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VI-5-849

Potential of mucosal *in vitro* models obtaining approval for drug products – case studies with Caco-2 and Calu-3 cells

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Drug development is growing more complex and time consuming. Furthermore, the generic market is growing and known drug molecules are formulated to improve therapy. Therefore, smart concepts should be integrated into the pharmaceutical development to avoid clinical studies to show bioequivalence of drug products. *In vitro* cell culture studies prove to be a fast and cost-effective tool to assess formulation influences on the bioavailability of a drug molecule. For orally applied drugs the BCS system can be used for class 1 substances. An example will be shown that this system can also be applied to other than fast releasing oral tablets as wells to non-class I substances to get approval.

The scientific base of the BCS system can also be applied to local acting substances, e.g., Corticosteroids against allergic rhinitis. Calu-3 cells as a robust mucosal model can be used to show bioequivalence of such products. An example of *in vitro* BE will be presented. The data show clearly that in *in vivo/in vitro* correlation cam be established and furthermore that the European Authorities accept in vitro also for the approval of drug products.

VI-5-864

Application of *in vitro* human airway models for toxicology, drug delivery and disease modeling

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Organotypic *in vitro* human airway models offer the opportunity to screen for potential airway toxicity of nanoparticles and chemicals, screen the safety and efficacy of active pharmaceutical ingredients and formulations intended for inhalation delivery, and model airway infection by bacterial and viral pathogens. Here we present a survey of successful uses of a commercially available *in vitro* human airway model (EpiAirway™) for these applications. The EpiAirway™ model is produced by culturing normal human airway epithelial cells on microporous membrane inserts at the air-liquid interface. The model reproduces the organotypic, pseudostratified mucociliary phenotype of *in vivo* proximal airways. EpiAirway™ model tissues have functional tight junctions, *in vivo*-like barrier properties and *in vivo*-like xenobiotic metabolizing capabilities. Specific examples surveyed from the published literature include toxicological evaluation of tobacco products, optimization of nasally administered therapeutics, long-term host-pathogen interactions during bacterial (*Haemophilus influenzae*) infection, mechanisms of influenza virus airway infection, and rhinovirus infection of asthmatic compared to non-asthmatic tissues.

VI-5-908

Modelling mucosal epithelia in state of inflammation

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For assessing the safety and efficacy of (nano)pharmaceuticals and other products, there is a need to study their interaction with mucosal barriers not only in healthy, but also in diseased state, i.e., when their barrier function might be impeded, especially in inflammation.

Regarding the oral route, a 3D model of the inflamed intestinal mucosa was developed by growing intestinal epithelial cells (Caco-2) on a collagen layer, comprising immune cells (dendritic cells: Mutz-3; macrophages: THP-1). After stimulation with interleukin-1β, this model allowed the assessment of anti-inflammatory drugs and nanomedicines (Leonard et al., 2010, 2012).

Considering the importance of aerosol exposure/delivery to the lungs, we translated this approach to a model of the air-blood barrier, consisting of human alveolar epithelial cells (hAEpC), that differentiate into type I cells and show pronounced barrier properties (Daum et al., 2012). Autologous co-cultures of hAEpC and alveolar macrophages originating from the same patient can be stimulated with lipopolysaccharides (LPS) to study particle uptake and anti-inflammatory drug effects. However, in addition to these cellular elements of mucosal barriers, non-cellular elements, such as in particular mucus and surfactant, must not be neglected, as they act as utmost efficient clearance and diffusional barriers and may form a specific lipid-protein corona especially for nanoparticles deposited in the respective parts of the lungs (Kirch et al., 2012; Ruge et al., 2013).

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VI-5-932

In-vitro models of the oro-gastro-intestinal mucosa

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The oral cavity, although part of the aero-digestive tract, is still neglected in terms of risk assessment with respect to nanoparticle uptake (Teubl et al., 2014; Teubl et al., 2013a. If nanoparticles enter the oral cavity, either via oral products or inhaled materials, it is not clear, whether they first interact with the mucosae or are swallowed and end up in the small intestine. To learn more about this relationship *ex-vivo* and *in-vitro* models that closely mimic the oral cavity and the small intestine were developed in order to study the spatio-temporal aspects of nanoparticle uptake, as well as the intracellular localization in human epithelial cells and potential toxic effects.

For this, buccal/sublingual human cells (TR146) were incubated with an external (human/porcine) mucus layer, prepared by a film method (Teubl et al., 2013b). Mucin fibres adhered to the stratified squamous epithelium and the viability of the model was maintained for more than 48 hours. Nanoparticle uptake rates correlated well with data from *ex-vivo* permeability studies through porcine buccal tissue (Roblegg et al., 2012).

In the small-intestine study, co- and triple culture models were established, mimicking the epithelium of villi (i.e., Caco-2 cells and goblet cells) and the follicle associated epithelium (i.e., Caco-2 cells, goblet cells and M cells), respectively (Schimpel et al., 2014). The data revealed that the mucus layer, which entirely covers the enterocytes, prevents penetration of conventional nanoparticles to the epithelial surface and that particle uptake in the small intestine mainly occurs via M cells.

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VI-5-933

Potential and limitations of reconstructed human skin models in assessing pharmaceutical formulations

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In vitro methods are being validated and adopted by regulatory agencies for the use as full or partial replacements of animal experiments. To date, the best results for topical toxicity testing *in vitro* have been obtained with reconstructed human skin models (RHSM). Particularly for the determination of skin corrosion and skin irritation, RHSM are the most predictive tools *in vitro*. Their use is also included in the OECD Test Guidelines (TG 431 (OECD, 2004) and 439 (OECD, 2013)), in the EU regulatory framework for chemicals REACH (2006) and recently, also ICH guideline (ICH, 2013) implemented use of the RHSM tissues for phototoxicity assessment of the topically applied substances.

RHSM are usually seen as reliable tools in assessments of chemical hazard, however, their use can be extended also into the area of risk assessment process and preclinical safety evaluation if appropriate protocols are developed as demonstrated. Also in the area of medical devices, the reconstructed tissue models are considered as useful tools in the assessment of human skin irritation effects (Casas et al., 2013).

The presentation will review the use of the RHSM models for *in vitro* assessment of pharmaceutical formulations. Recently published data in the area of irritation, phototoxicity, skin penetration, skin metabolism, genotoxicity and wound healing will be discussed.

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Session VI-5: Poster presentations

VI-5-281

Establishment of an intestinal model for oral vaccination testing

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The intestinal epithelium forms an impenetrable barrier for macromolecules and microorganisms, except the follicle-associated epithelium of the gut-associated lymphoid tissue where microfold (M) cells sample the intestinal lumen initiating an immune response. This entry site to the immune system is an interesting target not only for pathogens but also for the analysis and improvement of oral vaccines.

In this work, a functional intestinal *in vitro* model based on the adenoma carcinoma cell line Caco-2 co-cultured with dendritic cells derived from human blood on a three-dimensional matrix from acellularized porcine jejunum (SIS-Muc) was established.

In a proof-of-concept experiment the 3D co-culture model could demonstrate an activation of the dendritic cells with Lipopolysaccharides over the epithelial barrier observed by the upregulation of maturation markers (CD80. CD83, CD86) and interleukin 6 production, measured via FACS and ELISA analyses. Additionally, transmission electron microscopy was used to visualize M cells by the presents of truncated microvilli.

Taken together an improved three-dimensional intestinal *in vitro* model suited for infection and oral vaccination studies could be established which can be further improved and modified for specific issues. In this respect, we are currently using autologous human primary cells, which is of particular importance for future vaccination studies.

VI-5-299

Full-thickness tissue engineered development using human keratinocyte and adipose tissue derived mesenchymal stem cells

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Tissue engineering is an emerging therapeutic strategy that has the potential to revolutionize the skin regeneration treatments (Xie et al., 2007). Three-dimensional epidermis has been previously reconstituted *in vitro* using keratinocytes and adipose tissue derived mesenchymal stem cells (ADSC) seeded on dermal substrates in an air-liquid (A/L) environment (Lu et al., 2012). ADSCs present immunomodulatory potential and are easily accessible (Du et al., 2010; Sheng et al., 2013). In addition, these cells have can differentiate into fat, bone, cartilage, and muscle under lineage-specific culture conditions (Tholpady et al., 2006). The aim of this study was to develop a three-dimensional full-thickness engineered skin substitute using irradiated glycerol-preserved acellular dermis as support for the proliferation of different numbers of keratinocytes and ADSCs. Our results show that 300,000 keratinocytes cell/cm^2 and 300,000 ADSCs cell/cm^2 plated at the same time are enough to create a full-thickness dermal-epidermal substitute.

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VI-5-354

HuIFN-αN3 and 10% PBS holocene grain wash-out affect the glutathione and lipid peroxidation in CaCo-2 cells

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Purpose: HuIFN-αN3 and 10% PBS (Phosphate Buffer Saline) Holocene grain washout affects growth and apoptosis of CaCo-2 cells (Kesteli et al., 2007; Filipič, 2009). The purpouse of experiments was

to measure the effect of HuIFN-α and 10% PBS washout of holocene grain on the on the Glutathione level and Lipid peroxidation in CaCo-2 cells.

Methods: HuIFN-αN3, HuIFN-α2a, HuIFN-α2b and 10% PBS Holocene grain washout were used. The cells were treated : (a) Cell control, (b) HuIFN-α + 10% PBS (1:1.1:2.2:1), (c)HuIFN-α, (d) 10% PBS. The AP_{50} inhibiton test was used. Apoptosis was measured by "BioVision: Apoptotic cell isolation kit". Glutathion and Lipid peroxidation were determined as descibed (Devasagayam et al., 2000).

Results: 10% Holocene grain washout AP_{50} activity can be enhanced by HuIFN-αN3, but not with the HuIFN-α2a or HuIFN-α2b. 10% Holocene grain washout show 26.52% of apoptotic cells, while this % was increased to 49.85 with HuIFN-αN3. In the combination between HuIFN-αN3 and 10% PBS Holocene grain washout 2:1 the level of GSH was 24.9 ±2.4 nM/mg (70.2 ±3.2 nM/mg in control) and level of MDA 72.3 ±3.1 nM/mg (23.6 ±9.1 nM/mg in control).

Conclusion: HuIFN-αN3 and 10% PBS holocene grain wash-out synergize but not HuIFN-α2a or HuIFN-α2b in effect on Glutathione level and Lipid peroxidation.

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VI-5-569 *

Evaluating reconstructed cutaneous squamous cell carcinoma

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Non-melanoma skin cancer (NMSC) is the most prevalent cancer; lethal progressions affect immunocompromised patients (Stockfleth et al., 2008). Drug treatment of multiple or subclinical lesions lacks of sustainable efficacy and tolerability. The low predictive value of animal-generated data for humans (Soek et al., 2013) contributes to the failure of investigational new drugs in clinical trials (70% for all indications; Hartung et al., 2013). Although reconstructed human skin has been available for decades, commonly accepted NMSC models rely on animal-based systems. In this study, we evaluated the histopathology of our organotypic NMSC model.

To reconstruct NMSC, we co-cultured human squamous cell carcinoma cells (SCC-12) with juvenile normal human keratinocytes on a dermal equivalent consisting of normal human dermal fibroblasts (Ali-von Laue et al., 2014). For comparison, normal reconstructed human skin was built without SCC-12 cells. The variation of the seeding ratio, normal human keratinocytes to SCC-12 cells, determined the tumor stage from carcinoma *in situ* to the invasive disease. Tumor nests with atypical, dysplastic cells show increased Ki-67 index, borders with collagen-IV expression, reduced keratin-10 and keratin-14 expression.

In conclusion, our organotypic NMSC model shows several disease hallmarks. Further studies will focus on the evaluation of biomarkers to introduce this organotypic model into nonclinical drug development as a long-term goal.

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VI-5-822

Evaluation of serum growth factors in wound healing using a full-thickness *in vitro* human skin model

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Following skin injury, damaged tissue undergoes highly coordinated biological events to restore barrier function involving cross-talk between dermal fibroblasts and epidermal keratinocytes as well as their interaction with the extracellular matrix. A full-thickness *in vitro* human skin model (EpiDerm-FT™) was used to evaluate the role of serum growth factors in cutaneous wound healing. This model is constructed from primary keratinocytes and fibroblasts and contains a functional barrier and fully developed basement membrane. Small epidermal-only wounds (3mm biopsy punch) or full-thickness wounds (cauterizer burns) were induced in the tissue model and monitored histologically from day 0 to day 6. Addition of 2% human serum demonstrated an increased rate of epithelial healing and fibroblast accumulation which could be abrogated in the presence of an EGFR tyrosine kinase inhibitor or a TGF-alpha neutralizing antibody. Gene expression analysis of the wounded area showed temporally regulated changes in mRNA expression of basement membrane components, collagens and genes involved in extracellular matrix remodeling on days 2, 4 and 6 post wounding. These results demonstrate that EpiDerm-FT is a useful *in vitro* skin model for investigating dermal-epidermal interactions during wound healing as well as for the evaluation of new therapeutics in the dermal wound healing process.