

Alternatives to animal testing in basic and preclinical research of atopic dermatitis

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Funding information

Berlin Brandenburg 3R Research Platform

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease of increasing prevalence, especially in industrialized countries. Roughly 25% of the children and 1%-3% of adults are affected. Although significant progress has been made in the understanding of the pathogenesis of AD, many aspects remain poorly understood. Moreover, there is a pressing need for improved therapeutic options. Studies to elucidate the pathophysiological pathways of AD and to identify novel therapeutic targets over the last few decades have been conducted almost exclusively in animal models. However, in vitro approaches such as 3D skin disease models have recently emerged due to an increasing awareness of distinct interspecies-related differences that hamper the effective translation of results from animal models to humans. In addition, there is growing political and social pressure to develop alternatives to animal models according to the 3Rs principle (reduction, refinement and replacement of animal models).

KEYWORDS

3R approach, atopic dermatitis, preclinical dermatology, skin equivalents, skin model

1 | INTRODUCTION

Atopic dermatitis (AD) is a major public health problem. Currently, it affects up to 25% of the children and 1%-3% of adults in the Western world, although these figures continue to rise, likely due to cleaner living conditions (= hygiene hypothesis) especially in urban areas.^[1] AD is a multifactorial disease: besides genetic predispositions, environmental and immunological factors considerably contribute to the disease manifestation and pathogenesis. Defects in the epidermal barrier of human skin favour sensitization and strong inflammatory responses to external stimuli that ultimately trigger systemic responses.^[2] This barrier defects may be inherited. Prominent examples include mutations in the filaggrin gene identified in 10%-50% of AD patients and are a major predisposing factor.^[3] More than 50% of AD patients with moderate to severe phenotype undergo the atopic march, the concomitant sensitization to indoor-, then later outdoor allergens that progress to asthma and allergic rhinitis.^[4,5] Although all features of the atopic march are associated with a high physical and socio-economic

burdens, the molecular mechanisms that underlie the condition remain elusive, a knowledge gap that has hampered the development of new concepts for the treatment and prevention of atopic diseases.

Although significant progress has been made, the initial trigger for AD as well as its mechanisms of the progression is also still largely ambiguous. This is likely the result of not only the complexity of the disease, but also the lack of adequate disease models.

Animal models, particularly mouse models, are still the gold standard for basic research in AD, regardless whether the pathophysiology or novel therapeutic approaches are studied.^[6] However, unlike humans, normal mice do not develop AD spontaneously. Moreover, recent gene analysis has shown that while human and mouse skin share some features, only ~30% of skin-associated genes overlap between the two species.^[7] Indeed, major differences were detected in genes associated with skin morphogenesis, growth and immunology. In addition, the anatomy of rodent skin differs markedly from humans in, for example, the number of epidermal cell layers and hair bulbs as well as the presence of an additional muscle layer in mice, the *panniculus carnosus* (Figure 1).^[8,9]

Speaking more generally, there is accumulating evidence that the impact of interspecies-related differences has long been

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underestimated. About 80% of potential therapeutics fail in clinical trials, despite clear efficacy and safety in preclinical animal studies.^[10] This is not only expensive but also dangerous, especially to clinical trial subjects suffering from life-threatening diseases. The impact of interspecies-related differences has been highlighted in several publications^[11,12] including Seok et al^[13], who demonstrated the lack of predictivity of mouse models especially for inflammatory diseases. The reader should, however, note the latter study is controversially discussed. In particular, Takao and colleagues re-evaluated the same data set as used by Seok et al with a different statistical approach and came to the opposite conclusion, that there was an excellent correlation between mice and humans.^[14] In the very least, these two publications highlight the difficulty and caution that is required in correctly deriving information from mouse studies for the human situation.

The necessity for better, ideally human-based models in basic and preclinical research cannot be ignored on the grounds of disease accuracy, relevant therapeutic development and animal welfare. There is increasing ethical pressure to improve the rights and welfare of research animals that, within the European Union, is pushing the development of valid alternative test systems. For example, the general ban on the sale of cosmetic products tested in animals that came into effect as of March 2013 across Europe. As a consequence, cosmetic companies have been forced to increase their efforts to develop alternative test methods.

With regard to the above, reconstructed human tissues have gained increasing attention for multiple applications.^[15] Nevertheless, the development of disease models is still in its infancy. With regard to skin, disease models are currently generated from patient-derived cells,^[16] by modulation of disease-associated genes,^[17] by co-cultivation with pathogens^[18] or malignant cells,^[19] or by addition of disease-associated stimuli such as cytokines.^[20]

In this review, we aim to provide a critical overview of established in vitro models of AD. Additionally, we also briefly summarize frequently used in vivo models and discuss their drawbacks. In the final sections, we discuss the limitations of the in vitro models and try to highlight critical aspects that require further improvement.

2 | ANIMAL MODELS OF ATOPIC DERMATITIS

Animal models are still the gold standard in dermatological research. A great variety of animal models of AD are in current use. Notably, dogs, cats and horses develop AD-like lesions spontaneously.^[21] Of these, canine AD models are frequently used as their phenotype and clinical symptoms closely resemble those of human AD.^[22] Irrespective of the fact they do not spontaneously develop eczematous lesion, mice are the most commonly used AD models because of their relatively low costs, short time to maturity and highly tractable genetic manipulation.^[23,24]

One of the most frequently used models is the *NC/Nga* mouse.^[25] Housed under conventional conditions, they spontaneously develop

skin lesions within 6-8 weeks after birth,^[25] which is not the case when kept under air-regulated specific pathogen-free conditions. The lesions closely resemble human AD clinically and histologically.^[26,27] Another "spontaneous" AD model is the flaky tail (*ma/maFlg^{fl/fl}*) mouse which express mutations in the genes of both filaggrin and matted, both of which contribute to the development of the atopic-like skin lesions.^[28,29] Mice of the *FLG^{fl/fl}* genotype express an abnormal pro-filaggrin polypeptide that does not form normal keratohyalin granules and is not proteolytically processed to filaggrin. As such, functional filaggrin is absent from the epidermal cornified layer.^[30] This model is of interest to study the role of filaggrin in AD and/or ichthyosis vulgaris, both of which characterized by reduced or even complete loss of filaggrin. Nonetheless, as the loss of filaggrin is not complete in flaky tail mice, the precise role of this protein cannot easily be determined in this model. Hence, one Japanese group has developed a filaggrin-null mouse,^[31] which, however, is not easily accessible.

Other approaches use repeated topical application of haptens such as oxazolone and trinitrochlorobenzene over an extended period to induce allergic contact dermatitis in hairless mice. Hapten-induced AD mouse models are frequently used as they are easily reproducible and cheap.^[32-34] A clear drawback of this model, however, is the long disease induction phase (up to 4 weeks). Moreover, AD-like skin lesions are induced artificially, bringing into question their predictive power for the human situation. While transgenic mice overexpressing or lacking AD-relevant mediators such as IL-4 or TSLP (thymic stromal lymphopoietin) are useful for the study of how single genes/proteins impact upon skin homeostasis, in general, they emulate only a very limited part of the disease.^[23,35]

Each of these models exhibits distinct differences from human AD. Moreover, their use is hampered by limited reproducibility, requirements for repeated pretreatments, a long prodromal time or restricted availability.^[24,36] Moreover, there are still the problems of interspecies-related differences. A recent study found distinct disparities between the transcriptomic profile of murine AD models and human AD.^[12] Here, gene analyses of six murine AD models were compared to human AD. Homologies to human AD were poor, the best being IL23-injected (37% homology), *NC/Nga* (18% homology) and oxazolone-challenged

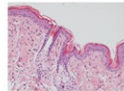
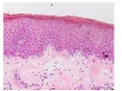
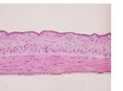
	Mouse	Human	Skin model
Histology			
Epidermal layers	2 - 5	16 - 18	10 - 15
Skin renewal	8 - 10 d	28 d	No
Hair follicle	High number & wider diversity	Significantly less than rodents	No
Cutaneous muscle layer	Panniculus carnosus	No	No
sweat glands	Eccrine sweat glands (exclusively in the pads)	Apocrine & eccrine	No
Melanocytes	Follicular location	Interfollicular location	Requires implementation
T-cell population in the skin	Dendritic epidermal T-cells (DETC)	α/β T-cells	Requires implementation
Antimicrobial peptides	Tissue specific different from humans	Yes	Similar to humans

FIGURE 1 Differences and similarities between mouse skin, human skin and human-based skin models

(17% homology) mice. Although only mRNA expression was analysed in this study and some bias may have been introduced by the selection of the human samples, the differences between mice and human are still remarkably large. To overcome these limitations, systematic data analysis such as gene set enrichment might be helpful to identify the most suitable animal model as it has been recently demonstrated for septic mouse models.^[37] This is not only improving the validity of the models but also a refinement approach according to the 3Rs principle.

3 | ALTERNATIVE METHODS IN RESEARCH ON ATOPIC DERMATITIS

3.1 | 2D Cell Culture and Co-culture

To study basic aspects of AD, classic monolayer culture of cells involved in the pathogenesis of AD such as keratinocytes or immune cells may be sufficient. An interesting example is the cytokine profiling of primary keratinocytes isolated from unaffected skin of AD patients.^[38,39] Compared to healthy donors, higher levels of granulocyte macrophage colony-stimulating factor (GM-CSF) was found in the AD keratinocytes. Interestingly, when culturing peripheral blood mononuclear cells (PBMCs) in the conditioned medium of AD keratinocytes, PBMC proliferation was induced in a GM-CSF-dependent manner indicating that keratinocytes themselves may regulate inflammatory responses.^[39]

Furthermore, the effect of specific cytokines on keratinocytes from AD patients can be studied in 2D culture. In this course, the Th cell-derived cytokines IL-4, TNF- α and IFN- γ were reported to affect the production of the pro-inflammatory chemokines IL-8, RANTES or monocyte chemoattractant protein 1 in keratinocytes.^[38] In a similar study, keratinocytes were stimulated with Th2 cytokines such as IL-4 and IL-13, which reduced filaggrin expression,^[40,41] well in line with the clinical situation of lesional atopic skin.^[42]

To study the role of immune cells such as lymphocytes, mast cells or eosinophils, gene expression and protein levels of these cells when isolated from peripheral blood or AD patient skin biopsies have been compared.^[43–46] Using this approach, a correlation between the severity of AD and the frequency of T cells expressing TSLP receptors was described.^[46] Moreover, it was demonstrated that the regulation of mast cells and their production of IgE is not affected by genetic predisposition but rather by stimulation with interleukins such as IL-4.^[44]

To create a more complex environment, co-cultures of skin-derived and immune cells are suitable tools. For example, Jiao et al studied the interactions between dermal fibroblasts and basophils/eosinophils in the presence of the Toll-like receptor 2 ligand peptidoglycan, a cell wall component of *Staphylococcus aureus* known to exacerbate AD-like symptoms. Both immune cells stimulated the release of AD-related cytokines and chemokines. Interestingly, while crosstalk between eosinophils and dermal fibroblasts was mediated by soluble factors, crosstalk between basophils and fibroblasts required direct cell contact.^[43]

Although studies in 2D monolayers or co-cultures can provide important initial information, these models do not emulate the complex interplay between epidermal barrier function, skin cells and immune

cells closely enough. Moreover, several publications have indicated significant divergences between the 2D and 3D culture of the cells from several organs including the skin,^[47] lungs,^[48] liver,^[49] heart^[50] and the central nervous system.^[51] An overview of the differences between 2D and 3D models is given in Table 1.

3.2 | 3D Tissue models

3.2.1 | Epidermal models emulating characteristics of AD

Reconstructed human epidermis (RHE) models facilitate the examination of keratinocyte-specific cell responses and can help to estimate the influence of distinct inflammatory cytokines. The value of this approach has been demonstrated repeatedly. For example, Bernard and colleagues exposed RHE to a cytokine cocktail of IL-4, IL-13, TNF- α and IL-22 that induced an AD-like histology, decreased filaggrin expression and increased expression of antimicrobial peptides.^[52] Exposure to higher concentrations of IL-4, IL-13, TNF- α and IL-31 gave similar results and altered the levels of long-chain free fatty acids and ester-linked ω -hydroxy ceramides, crucial components of the lipid barrier of human skin.^[53] Concordantly, stimulation with a combination of TNF- α , IL-4, IL-13 and the Toll-like receptor 3 ligand Poly I:C^[54] resulted in differential expression of 26 genes including key markers of atopic skin. A further study included methyl- β -cyclodextrin, a molecule that extracts cholesterol from plasma membranes and thus disrupts lipid microdomains.^[55] The combination of membrane cholesterol depletion and the Th2 cytokines IL-4/IL-13 and IL-25 also elicited an AD-like phenotype. In fact, these data demonstrate the impact of pathophysiologically relevant mediators can be easily studied in vitro. Interestingly, in many instances, similar findings have been reported for AD patients.

In addition to the study of cytokine effects in epidermal layers, RHE can be also used to investigate the impact of genetic

TABLE 1 Advantages and disadvantages of in vitro 2D and 3D models

	2D cultures	3D tissue models
Structure	Simple monolayer or co-cultures, no stratification	Epidermal differentiation & maturation, stratification
Application	Initial studies on cell reactivity and cell-cell interactions	Investigation of more complex tissue-specific effects in normal and diseased states
Air-liquid interface	No	Yes, crucial for stratification
Dynamic cultivation	No	In principle possible, but improvements still required
Preparation & cultivation time	Short—few days	Up to several weeks
Costs	Low costs	High costs

modifications on the formation of the epidermal barrier. Here again, a point of interest is the filaggrin deficiency often found in atopic skin that, though widely discussed, remains poorly understood. Pendaries et al^[56] knocked down *FLG* and *FLG2* expression^[57] in RHE using shRNA induced RNA interference. The knockdown of *FLG* induced impaired epidermal differentiation and reduced natural moisturizing factor levels plus increased permeability and UV-B sensitivity.^[56] The function of *FLG2* in human skin is even less well characterized, although it was shown that *FLG2* expression is reduced in European AD patients, probably due to downstream effects of pro-inflammatory cytokines.^[58,59] *FLG2* knockdown triggered epidermal thinning, parakeratosis, increased skin surface pH (mean value from 6.38 ± 0.25 to 7.1 ± 0.15) and, again, increased sensitivity towards UV-B irradiation.^[57] Interestingly, many of these aspects have been previously described for AD patients.

3.2.2 | Full-thickness models emulating AD characteristics in vitro

In contrast to RHE, full-thickness (FT) models constitute a more complex skin model owing to the presence of a dermal compartment. The importance of keratinocyte–fibroblast crosstalk has been demonstrated repeatedly,^[60–62] although the underlying mediators and mechanism are still not fully understood.

The first FT model mimicking atopic skin in vitro was published by Mildner and co-workers, who developed a siRNA mediated filaggrin knockdown model.^[63] Shortly after, a similar approach was published by our group.^[64] While both studies reported reduced barrier function in the *FLG* knockdown models, K  chler et al also found inter- and intracellular spongiosis as well as impaired epidermal maturation and differentiation. That the latter was not observed by Mildner et al may be attributed to the different durations the FT were cultivated before assessment (K  chler et al, 14 days vs Mildner et al, 7 days). Further analysis of the 14-day model showed significantly reduced natural moisturizing factor levels.^[65] Interestingly, the surface pH of the normal and filaggrin-deficient skin models was physiological (~pH 5.5). This result was contrary to the long-held hypothesis that filaggrin played a crucial role in the maintenance of skin surface pH and that its reduced expression was key to the frequently increased surface pH of AD patients. In-depth analysis of the *FLG*-deficient models revealed a compensatory upregulation of sodium hydrogen antiporter (NHE-1) and phospholipase A₂ IIA that appeared to compensate for the lack of filaggrin.^[65]

Controversially, one study reported a *FLG* knockdown in which no changes were found in epidermal morphogenesis or SC lipid organization and composition, implying that a lack of filaggrin does not necessarily affect the skin barrier function.^[66] However, the authors used the keratinocyte cell line N/TERT instead of primary keratinocytes, a point that may limit the relevance of these results to human skin physiology.

Similar to epidermal models, the influence of Th2 cytokines IL-4 and IL-13 has been also studied in FT models. Kamsteeg et al^[67] stimulated a FT skin model with the Th2 cytokines IL-4/IL-13 and observed

morphological features similar to lesional atopic skin as well as increased expression of AD-associated genes. Furthermore, increased keratinocyte apoptosis was observed in the cytokine-stimulated models. H  nzke et al^[20] took this approach a step further, investigating the impact of the Th2 cytokines IL-4/IL-13 on filaggrin-deficient skin, an interaction that had not previously been addressed in AD research. Similar to atopic skin in vivo, skin models supplemented with Th2 cytokines demonstrated epidermal thickening and significantly increased skin surface pH (from 5.45 to 6.23) relative to normal FT models. In cytokine supplemented filaggrin-deficient models, the skin surface pH was increased even further (pH 6.37). This study highlighted the detrimental effects of Th2 cytokines in skin homeostasis, heightened in filaggrin-deficient as compared to normal models.^[20]

Further studies investigated the impact of other inflammatory mediators such as histamine, which also plays an important role in atopic diathesis. Gschwandtner et al^[68] exposed FT skin models to histamine for three days at different time points and found reduced expression of keratins, filaggrin and loricrin as well as the tight junction proteins occludin, claudin-1 and claudin-4. Notably, impaired epidermal differentiation was only observed when histamine was added to the tissue culture early on. In principle, histamine effects mediated by the H₁ receptor are believed to contribute to the transition from acute to chronic AD.

3.2.3 | Skin models emulating AD in vitro using patient cells

Very few studies have used AD patient cells, perhaps due to the personal reservations of the patients to the invasive procedure required to give the necessary skin biopsies, a sample normally not required for the diagnosis of AD. Moreover, AD is highly heterogenic, which makes it difficult to find a homogenous patient cohort. Nevertheless, Berroth et al studied the role of fibroblasts in the pathogenesis of AD in FT skin models. Notably, impaired keratinocyte proliferation and terminal differentiation as a result of reduced involucrin, loricrin and filaggrin expression were observed in FT skin models generated from atopic fibroblasts.^[16] Interestingly, skin models grown from healthy fibroblasts and AD keratinocytes showed no epidermal alterations. Further analysis of AD fibroblasts showed decreased expression of leukaemia inhibitory factor (LIF). The addition of recombinant LIF ameliorated the pathological phenotype seen in the AD fibroblasts FT skin model, indicating the potential role of LIF in atopic skin and for keratinocyte–fibroblast crosstalk in general.

In addition to the skin barrier impairment, atopic skin is also characterized by pruritus and a correlation between pruritus severity and the peripheral nervous system is discussed. To address this subject, Roggenkamp et al investigated how sensory nerve fibres and keratinocytes communicate with each other using FT skin models built from skin cells of AD patients. Here, a collagen gel containing porcine dorsal root ganglia neurons was attached below the dermis equivalent. The authors observed epidermal thickening, increased neurite outgrowth and increased release of mediators modulating cutaneous inflammation indicating that crosstalk between cutaneous nerves and skin cells do indeed influence epidermal morphogenesis.^[69]

3.2.4 | Skin models containing immune cells

As the implementation of dendritic and Langerhans cells has proven tricky, many groups have instead focused on T cells. In 2005, the first skin model co-cultured with memory and effector T cells mimicking characteristics of atopic skin was published.^[70] Here, the keratinocyte cell line HaCat was seeded on dermal equivalents that were then placed on a collagen matrix containing T cells. Skin models exposed to activated T cells developed epidermal spongiosis, cell apoptosis and showed enhanced levels of pro-inflammatory cytokines and chemokines, as well as increased expression of intercellular adhesion molecule-1. A recent approach succeeded in the generation of filaggrin-deficient skin models containing T cells.^[71] Most interestingly, T-cell migration into the dermal compartment of the skin models only occurred in the filaggrin-deficient models. In-depth analysis identified the keratinocyte-cytokine TSLP as a direct inductor of T-cell migration. This is of particular interest since until this point, only T-cell stimulation by dendritic cells was known. Moreover, after exposure to filaggrin-deficient skin model, the T cells showed a shift from a Th1/Th17 to a Th2/Th22 profile. The latter study is another example how useful in vitro disease models can be to study novel aspects of diseases.

4 | LIMITATIONS OF IN VITRO MODELS AND NECESSARY IMPROVEMENTS

So far, excellent in vitro tissue models have been developed that can be either purchased commercially or are grown according to in-house protocols. In parallel, microfluidic organ-on-a-chip tissue cultures have gained increasing interest as organ chips allow the combination of several tissues on one device within a human-like environment including systemic-like circulation.^[72–74] In contrast to these advancements, the development of human disease models is still in its infancy. Here, research efforts need to be intensified as human disease models have a proven ability to closely mimic disease patterns in vitro and for studying pathophysiological aspects.^[16,53,65] Nevertheless, only few in vitro disease models are described and even fewer combine two or more tissues on a single platform. This is a clear biological drawback, as single reconstructed (diseased) tissues are limited in recapitulating effects that occur within complex in vivo environments. Hence, advancements of the multi-organ approaches are important, as these would allow the study of communication between (diseased) organs in a more complex setting.

Another drawback of the currently available tissue models is the lack of vasculature and their mainly static cultivation, resulting in no or low exposure of mechanical forces such as stretching or shear stress. The importance of mechanical forces for tissue development has been repeatedly demonstrated for several organs including skin, ligaments and arteries.^[75–77] This limitation can be addressed by advances in, for example, microfluidic cell cultures^[78] or the establishment of dynamic perfusion chambers.^[79] Approaches towards the inclusion of

vasculature include the generation of in vivo like vascularized skin equivalents with a perfused vascular network. This has been achieved by combining a biological vascularized scaffold based on a decellularized segment of porcine jejunum and a tailored bioreactor system. Here, the perfused vascular network was re-seeded with endothelial cells that could then be perfused with a physiological volume flow.^[80]

A clear advantage of animal models over currently available in vitro models is their complexity including systemic circulation, an immune system and full metabolic capacity. Although it remains unclear to what extent the immunological responses of animals can be extrapolated to the human situation, it allows the study of disease phenotypes or novel drugs in a living organism. Therefore, a major drawback of in vitro models is the lack of immune cells. As AD is an inflammatory skin disease, however, the implementation of immune cells such as dendritic or T cells is imperative. So far, several groups have tried to implement dendritic cells/Langerhans cells into skin models.^[81–83] To the best of our knowledge, none of these approaches resulted in a set-up that sufficiently emulated the human situations. Frequent hurdles are altered expression of cell surface markers, unstimulated migration of these cells and unphysiological responses to certain stimuli. These problems may arise from the use of cells derived from different donors, the combination of primary cells and cell lines or simply an unphysiological environment. Other groups, including ourselves, have successfully implemented T cells into skin models.^[71,84]

Another problem is the lack of comparability of the models. This is not specifically a problem of in vitro models but also holds true for any in vivo model. Many different protocols for skin (disease) models are available that distinctly differ in terms of cell types (primary or cell lines), supplements added to the differentiation medium, composition of the dermal compartment (if one is attached) and the cultivation time (Figure 2). This may result in contradictory and difficult to interpret results. One “ideal” in vitro model of AD is likely very unrealistic and a simple, unifying solution will, in our opinion, not be found. Nevertheless, it is highly recommended to favour primary cells over cell lines, as the latter differ distinctly from primary cells in proliferation rates, differentiation etc. The use of primary cells,

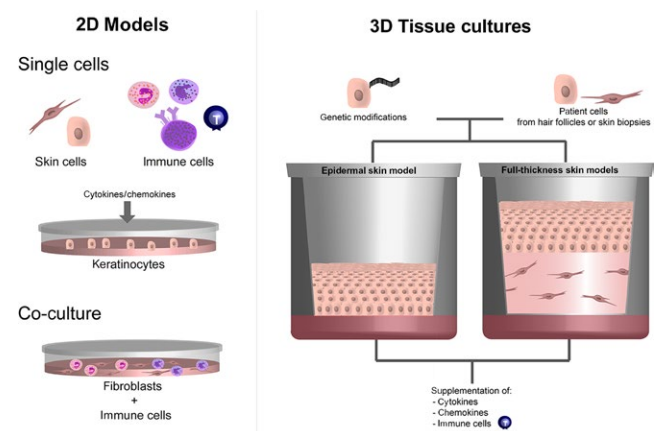


FIGURE 2 Overview of the different in vitro approaches in atopic dermatitis research

however, is associated with high batch-to-batch variabilities due to interindividual differences that may complicate the interpretation of data. Nevertheless, it displays the biodiversity of humans which can be also considered as a “plus.”

5 | CONCLUSION AND OUTLOOK

In summary, intensifying efforts in the development of AD disease models is a timely and necessary path. In vitro models mimicking AD have the potential to close a gap between preclinical and clinical studies and results obtained so far underline the value of in vitro models. Notably, as AD has high prevalence specifically in children, it would be interesting to investigate whether and how this can be mimicked in vitro. Nevertheless, despite the great advances that have been made, further model complexity such as the inclusion of a vasculature or immune cells is required. Moreover, similar to animal models, researchers have to pay great attention to the characteristics and limitations of their model, and an appropriate experimental design including adequate controls is imperative. A validation of the AD disease models against AD in humans would be favourable, but for most scientists this is difficult to realize.

ACKNOWLEDGEMENT

Financial support by the Berlin-Brandenburg research platform BB3R is gratefully acknowledged.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR'S CONTRIBUTION

The authors (S.H., A.L.) apologize to authors whose relevant contributions could not be cited because of space limitations.

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How to cite this article: Löwa A, Jevtić M, Gorreja F, Hedtrich S. Alternatives to animal testing in basic and preclinical research of atopic dermatitis. *Exp Dermatol.* 2018;00:1–8.
<https://doi.org/10.1111/exd.13498>