Pharmacological Research 194 (2023) 106848

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Manuka honey activates the aryl hydrocarbon receptor: Implications for skin inflammation

Abdullah A. Alangari [a,b,*,1], Matin D. Ashoori [b], Wisam Alwan [b], Hannah R. Dawe [b], Brigitta Stockinger [c], Jonathan N. Barker [b], Emma Wincent [d], Paola Di Meglio [b,**]

[a] Department of Pediatrics, College of Medicine, King Saud University, Riyadh, Saudi Arabia
[b] St. John's Institute of Dermatology, School of Basic and Medical Biosciences, King's College London, London, UK
[c] AhRimmunity Lab, The Francis Crick Institute, NW1 1AA London, UK
[d] Institute of Environmental Medicine, The Karolinska Institute, Stockholm, Sweden

A R T I C L E I N F O

Keywords:
Aryl hydrocarbon receptor (AHR)
CCL26
Filaggrin
IL-4
Manuka honey

A B S T R A C T

Manuka honey (MH) is a complex nutritional material with antimicrobial, antioxidant and anti-inflammatory activity. We have previously shown that MH down regulates IL-4-induced CCL26 expression in immortalized keratinocytes. As MH contains potential ligands of the Aryl Hydrocarbon Receptor (AHR), a key regulator of skin homeostasis, we hypothesize that this effect is mediated *via* AHR activation. Here, we treated HaCaT cell lines, either stable transfected with an empty vector (EV-HaCaT) or in which AHR had been stable silenced (AHR-silenced HaCaT); or primary normal human epithelial keratinocytes (NHEK) with 2% MH for 24 h. This induced a 15.4-fold upregulation of *CYP1A1* in EV-HaCaTs, which was significantly reduced in AHR-silenced cells. Pre-treatment with the AHR antagonist CH223191 completely abrogated this effect. Similar findings were observed in NHEK. In vivo treatment of the Cyp1a1^{Cre} x R26R^{eYFP} reporter mice strain's skin with pure MH significantly induced CYP1A1 expression compared with Vaseline. Treatment of HaCaT with 2% MH significantly decreased baseline CYP1 enzymatic activity at 3 and 6 h but increased it after 12 h, suggesting that MH may activate the AHR both through direct and indirect means. Importantly, MH downregulation of IL-4-induced CCL26 mRNA and protein was abrogated in AHR-silenced HaCaTs and by pre-treatment with CH223191. Finally, MH significantly upregulated *FLG* expression in NHEK in an AHR-dependent manner. In conclusion, MH activates AHR, both *in vitro* and *in vivo*, thereby providing a mechanism of its IL4-induced CCL26 downregulation and upregulation of *FLG* expression. These results have potential clinical implications for atopic diseases and beyond.

1. Introduction

Honey is a complex nutritional material known for its therapeutic potentials since ancient times. Manuka honey (MH), a monofloral dark honey produced by honey bees feeding on *Leptospermum scoparium* growing as shrub in New Zealand and eastern Australia, is antimicrobial, antioxidant and anti-inflammatory and is approved in Europe, USA, and other countries for the treatment of traumatic and surgical wounds, first and second degree partial-thickness burns, as well as diabetic, pressure and venous stasis ulcers [1–3]. Recently, MH has been shown to downregulate IL-4-induced production of the potent eosinophil chemoattract CCL26 in HaCaT keratinocytes, and to ameliorate erythema, edema and excoriation in a proof-of-principle clinical study involving 14 atopic dermatitis patients [4]. Thus, there is an emerging interest in MH as a potential therapeutic beyond wound care [5,6].

Despite the clear clinical benefit, the molecular mechanisms underpinning the beneficial effects of MH are poorly understood, possibly due to its complex composition. Honey consists of carbohydrates, minerals,

Abbreviations: MH, Manuka Honey; AHR, Aryl Hydrocarbon Receptor; FICZ, 6-formylindolo[3,2-*b*]carbazole; CYP1A1, Cytochrome P4501A1; EROD, 7-ethoxyresorufin-o-deethylase; NHEK, Normal Human Epithelial Keratinocyte.

* Correspondence to: P.O. Box 2925, Riyadh 11461, Saudi Arabia.
** Correspondence to: St. John's Institute of Dermatology, Guy's Hospital, King's College London, London SE1 9RT, UK.
E-mail addresses: aangari@ksu.edu.sa (A.A. Alangari), paola.dimeglio@kcl.ac.uk (P. Di Meglio).
[1] ORCID 0000-0002-0104-8175

https://doi.org/10.1016/j.phrs.2023.106848
Received 6 December 2022; Received in revised form 30 June 2023; Accepted 4 July 2023
Available online 5 July 2023
1043-6618/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

proteins, fatty acids, and other biologically active substances [7]. Among them, flavonoids, polyphenolic hydrocarbons, and indoles can activate the aryl hydrocarbon receptor (AHR) [8,9]. AHR is a transcription factor widely expressed in the body, including the immune system and epithelial cells of barrier organs, such as keratinocytes in the skin. In homeostatic conditions, AHR is located in the cytosol; upon ligand binding it translocates into the nucleus where it binds to response elements on the DNA regulating expression of multiple genes including the detoxifying enzyme cytochrome P4501A1 (*CYP1A1)* which -metabolises physiological AHR ligands such as the tryptophan derivative 6-formylindolo[3,2-*b*]carbazole (FICZ), thereby providing a negative feedback mechanism [10]. Activation of AHR signaling has been shown to be beneficial in both autoimmune and allergic skin inflammation, damping the production of pro-inflammatory cytokines and chemokines and enhancing skin barrier integrity [11,12], as well as in other experimental models of inflammation [13,14]. In particular, AHR activation by coal tar also downregulated IL-4-induced CCL26 [15]. Therefore, we aimed to investigate whether MH exerted its anti-inflammatory effects in the context of allergic skin inflammation, *via* activation of the AHR pathway.

2. Methods

2.1. Human subjects

Discarded healthy skin for isolation of primary keratinocytes was obtained from donors undergoing plastic surgery procedures at Guy's and St. Thomas' Hospital, London (UK). Full demographics can be found in Supplementary Table 1. Our study was conducted in accordance with the Helsinki Declaration, with written informed consent obtained from each volunteer, and approved by the institutional review board of Guy's and St. Thomas' Hospital (06/Q0704/18).

2.2. Mice

Cyp1a1^{Cre} x R26R^{eYFP}, reporting the expression of CYP1A1, were previously described [16,17]. Mice were bred in the Francis Crick Institute animal facility under specified pathogen free conditions and transferred to King's College London's animal facility for the experiments. All animal procedures were conducted under Project Licences granted by the UK Home Office.

2.3. Cell culture and exposure

HaCaT cells, either stably transfected with an empty vector (EV) or in which AHR had been stably silenced by 70% (AHR-KO)[18], were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (Thermofisher) to a final concentration of 10%, 100 IU penicillin and 0.1 mg/ml streptomycin mix (1% v/v), and 2 mM glutamine (1% v/v). Serum free DMEM was used in all experiments.

Normal human epithelial keratinocytes (NHEK) were isolated from discarded healthy skin as previously described [19] and routinely cultured in KGM medium (KBM medium, supplemented with KGM-bullet kit, both form Lonza). KBM medium was used in all experiments. Cells were used between passages 3–6.

Cells were treated with manuka honey (MH) UMF 10 + (Comvita, Maidenhead, UK) 0.02–2% wt/v in DMEM, filtered through a 0.22 µm syringe filter (Durapore), with/without addition of up to 100 nM FICZ (Enzo life sciences or Syntastic) in DMSO. Maximum concentration MH of 2% was selected based on viability assay in keratinocytes as previously described [4]. For these experiments, medium only or medium containing 0.1% DMSO was used as negative control. In some experiments, cells were treated with 2% English borage (Wilkin and Sons Ltd., Tiptree, UK), Organic Forest (Wainwright, Aberystwyth, UK), Rowse acacia and Rowse organic (Wallingford, UK). Human IL-4 (Milteny Biotech) was used at final concentration of 50 ng/ml 2 h post MH treatment. CH-223191 (Sigma-Aldrich) was used as AHR-selective antagonist 2 h prior to MH/FICZ treatment at a final concentration of 3 µM in DMSO. Cell supernatant was collected in specific experiments and stored at − 80 until further use. Adherent cells were washed with PBS, detached with trypsin, washed with ice cold PBS and the dry cell pellet was collected and stored at − 80 until further use.

To investigate AHR nuclear translocation, HaCaT cells were seeded at a density of 7×10^3 cells per well in Nunc Lab Tek II 8-well Chamber Slides (Thermofisher) until 70% confluency. Cells were either pretreated with 3 µM CH or left untreated for 2 h, and then were treated with FICZ, increasing concentrations of MH (0.02%, 0.2%, 2%), or 0.02% DMSO as vehicle control for 30 min. The cassette was then removed, and the slides were subjected to immunofluorescence staining.

2.4. RNA extraction and RT-qPCR

RNA was extracted using RNeasy plus mini kit (Qiagen), as per manufacturer's instructions, quantified by NanoDrop (Thermofisher), and 250 ng were converted into cDNA using high-capacity cDNA reverse transcription kit (Thermofisher, UK). qPCR was performed using TaqMan gene expression master mix (Thermofisher) and Taqman primers and hydrolysis probes for *CYP1A1* (Hs01054974_m1), *CCL26* (Hs00171146_m1), *FLG* (Hs00856927_g1), and *RPLP0* as reference gene (Hs99999902_m1). Results were expressed as either relative mRNA expression calculated using the ΔCq method or as fold change to the negative control used as calibrator (medium only for MH or 0.1% DMSO for FICZ and FICZ+MH), using the ΔΔCq method.

2.5. Ethoxyresorufin-O-Deethylase (EROD) Assay

CYP1A1 activity was measured using the EROD assay as previously described [10]. Briefly, HaCaTs were seeded in quintuplicates in 96-well transparent plates at 5×10^4 cells/well. Overconfluent cells (one day after reaching 100% confluence) were washed with PBS and treated with either MH, FICZ, MH+FICZ or DMSO in serum free DMEM for 3, 6, 12, 24 or 48 hours. Exposures were terminated at the indicated time points by removing the medium and rinsing the cells with PBS and the EROD reaction was initiated by adding 100 µl ethoxyresorufin (2 µM, Sigma-Aldrich) in sodium phosphate buffer (50 mM, PH 8.0) to each well. The cells were incubated at 37° C for 20 min and the reaction was stopped by adding 75 µl fluorescamine in acetonitrile (150 ug/ml, Sigma-Aldrich) per well. Resorufin formation was measured against a standard curve with excitation/emission wavelengths 544/590 (FLUOstar Omega, Labtech). CYP1A1 enzymatic activity was normalized for the cellular protein content determined by fluorescamine fluorescence of a standard curve of bovine serum albumin at excitation/emission wavelengths 390/480.

2.6. CYP1 enzyme inhibition assay

Effects of MH on CYP1A1 activity was determined using human recombinant CYP1A1 (2.5 nM, Sigma Aldrich). First, the enzyme was preincubated with respective concentration of MH for 5 min in Tris-HCl (0.1 M, pH 7.4) with EDTA (1 mM) at 37 °C, followed by addition of the CYP1 substrate ethoxyresorufin (0.1 µM). The suspension was transferred in triplicates to a white 96-well plate and the reaction was initiated by adding NADPH (1 mM, Sigma Aldrich). Formation of resorufin was monitored every 2 s using a multiwell plate reader (infinite F200 PRO, Tecan) with the excitation/emission wavelengths of 535/590 nm. CYP1A1 activity was determined by the rate of resorufin formation during the first 90 s of the reaction, and percentage of inhibition was calculated relative to the enzymatic activity observed without addition of MH. Percentage of MH causing 50% inhibition of activity (IC50) was calculated by nonlinear regression modelling (variable slope, four parameters, GraphPad Prism Software).

2.7. ELISA

CCL26 protein was measured in cell supernatants using Quantikine ELISA system (R&D Systems, Cat# DY346) as per manufacturer's instructions.

2.8. In vivo treatment with MH and CYP1A1 evaluation

Cyp1a1^{Cre} x R26R^{eYFP} reporter mice (8–10 weeks old, males and females) were shaved and topically treated on the back with vaseline as negative control (150 mg/mouse, Unilever), or MH (150 mg), or FICZ as positive control (5 μg in 50μl corn oil) [17] daily for 5 consecutive days. On day 6, skin was excised and cryopreserved in OCT (VWR). Skin sections were prepared and fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X in PBS, and non-specific binding was blocked with 30% rabbit serum. Slides were stained with, Alexa Fluor-594 anti-GFP (ThermoFisher Scientific) for 45 min at room temperature and subsequently counterstained with DAPI (ThermoFisher Scientific). Slides were imaged using a Nikon Eclipse Ti-2 Inverted Microscope. Images were processed using Fiji ImageJ, applying the same processing methodology to all images. Intensity of YFP expression in the epidermis was measured in three regions of interest (ROI) per slide and expressed as arbitrary fluorescence unit.

2.9. AHR immunofluorescence staining

HaCaT cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X in PBS, and non-specific binding was blocked with 10% normal goat serum. Cells were stained overnight with a polyclonal sheep IgG anti-human AHR antibody (15 μg/ml; R&D Systems) at 4 °C. Next, cells were incubated with anti-sheep Dylight 488 (1:50 dilution; Vector) for 45 min at room temperature. Cells were then counterstained with DAPI (1 μg/ml) for 2 min at room temperature. Slides were then imaged using a Nikon Eclipse Ti-2 inverted microscope, with 5 fields of view (FOW) taken for each well at 10x magnification. Images were processed using Fiji ImageJ.

2.10. Statistical analysis

All *in vitro* experiments were performed at least in triplicate and data presented are means ± SE. Means were compared using one-way or two-way ANOVA as appropriate, followed by Sidak's or Tukey's multiple comparisons test, respectively. For evaluation of CYP1A1 expression *in vivo*, fluorescence values are expressed as the mean ± SEM of the number of ROI measured and statistically analysed with one-way ANOVA and Dunnet's test for multiple comparisons. The images shown are representative of at least 5 individual mice per group. Statistical analyses were performed with GraphPad Prism version 9. p value < 0.05 was considered significant.

3. Results

To evaluate if MH can activate the AHR pathway we used HaCaT keratinocytes, stably transfected with an empty vector (EV), or in which AHR had been stable silenced by 70% (AHR-KO HaCaT) [12,18]. Treatment of EV-HaCaT cells with 2% MH for 24 h induced a 15.4-fold up-regulation of *CYP1A1* mRNA expression over control medium (Fig. 1A). As expected, the prototypical AHR agonist FICZ also upregulated *CYP1A1* mRNA expression, while *CYP1A1* mRNA induction by either FICZ or MH was significantly less prominent ($p \leq 0.001$) in AHR-KO HaCaT cells (Fig. 1a). MH induction of *CYP1A1* was concentration-dependent, maintained over time, and completely abrogated by pre-treatment with the AHR antagonist CH223191 ($p \leq 0.0001$) (Fig. 1b). Similarly, 2% MH induced statistically significant expression ($p \leq 0.0001$) of *CYP1A1* in primary NHEK, which was completely abrogated ($p \leq 0.0001$) by pretreatment with CH223191

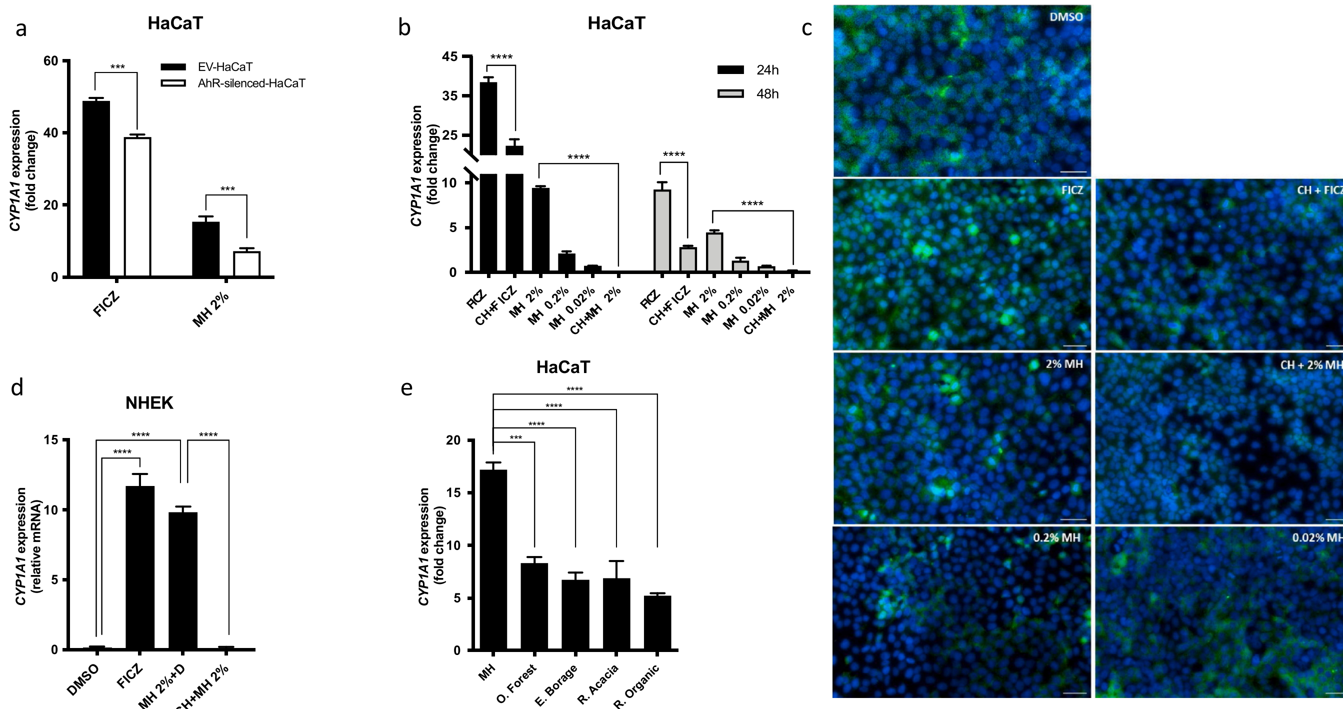

Fig. 1. a) *CYP1A1* mRNA fold change in empty vector (EV) HaCaTs and AHR silenced HaCaTs. b) *CYP1A1* mRNA fold change in EV HaCaTs. c) Representative images showing AHR nuclear staining following 30 min treatment. Scale bar is 50 μm. d) *CYP1A1* relative mRNA expression in NHEK. e) Comparison of *CYP1A1* mRNA expression mean fold change in empty vector (EV) HaCaTs treated with manuka honey (MH) and some other commercial honeys (all as 2% w/v). O. Forest: Organic Forest, E. Borage: English Borage, R. Acacia: Row Acacia, R. Organic: Row Organic. Two-way ANOVA with Sidak's (a,b,d) or one-way ANOVA with Tukey's (c) multiple comparisons test. MH: Manuka honey, D: DMSO, * ** $p < 0.001$, * ** * $p < 0.0001$.

(Fig. 1c). Interestingly, while honeys from different plant sources, including, 2% English borage, Organic Forest, Rowse acacia and Rowse organic, induced *CYP1A1* expression in EV-HaCaT, MH was significantly superior (2–3 times) in activating the AHR pathway (Fig. 1d).

To confirm the ability of MH to modulate the AHR pathway *in vivo*, we made use of the Cyp1a1^{Cre} x R26R^{eYFP} reporter mice strain [16,17]. Pure MH or FICZ were applied topically for 5 days on the back of the mice and CYP1A1 expression was evaluated on day 6. Minimal CYP1A1 reporter activity, mostly in the hair follicles, was observed in control mice treated with Vaseline (Fig. 2a); as expected [17] topical treatment with FICZ significantly ($p < 0.0001$) induced CYP1A1 expression in the epidermis and dermis (Fig. 2b). Importantly, MH also significantly ($p < 0.0001$) induced CYP1A1 expression in the skin of Cyp1a1^{Cre} x R26R^{eYFP} reporter mice (Fig. 2b).

A number of putative AHR ligands have been recently shown to be instead, inhibitors of CYP1 enzymatic activity [10], thereby indirectly activating the AHR pathway by reducing the metabolism of endogenous ligands. Thus, to further verify our findings, we evaluated the effect of MH on CYP1 activity over time using the EROD assay (Fig. 3a). Overall, an initial dose dependent CYP1 inhibition by MH was observed, followed by an activation. Treatment of HaCaT with 2% MH significantly decreased baseline activity at 3 and 6 h but increased it after 12 h and up to 48 h, while 0.5% MH did not exert any significant effect up to 3 h but displayed an increased activity at 6- and 12 h, suggesting the presence of both CYP1 inhibitors and AHR ligands. To confirm that MH is able to both inhibit CYP1 activity and activate AHR we cultured HaCaT cells in the presence of increasing concentrations of FICZ (5, 50, and 100 nM), with or without 0.5–2% MH, for up to 48 h (Fig. 3b, c and Supplementary Figure 1). As expected, FICZ significantly increased CYP1 activity over DMSO control at each timepoint, peaking at 6–24 h, in a dose-dependent manner. 2% MH significantly reduced the enzymatic activity induced by 50 nM and 100 nM FICZ at all timepoints (Fig. 3b and Supplementary Figure 1), further indicating the presence of inhibitors of CYP1 activity. When a 20-fold lower concentration of FICZ was used (5 nM, Fig. 3c), 2% MH only inhibited the effect of FICZ at the 3 h and 6 h time points, while significantly potentiating CYP1 activity at 12 and 24 h treatment. This appearance, with an initial inhibition followed by potentiated EROD activity is in line with previous data on the impact of CYP1 inhibitors on AHR signaling [10], suggesting that MH may activate the AHR both through direct- and indirect means. As cellular EROD inhibition may stem from either reduced expression of the CYP1A1 protein or inhibition of its activity, we next used recombinant CYP1A1 enzymes to confirm the inhibitory effects of MH on the activity specifically. As can be seen in Fig. 3d, MH did indeed inhibit the enzyme activity, with 50% inhibition observed with 0.19% MH.

Having convincingly shown that MH is able to activate the AHR pathway either directly or indirectly, we evaluated whether AHR was involved in MH ability to downregulate IL-4-mediated CCL26 induction. As previously shown [4], treatment of EV-HaCaT cells with IL-4 for 24 h significantly upregulated the release of CCL26 in the supernatant (Fig. 4a) and pre-treatment with MH significantly downregulated ($p \leq 0.0001$) IL-4-driven CCL26 release in a concentration-response fashion (Fig. 4a). Importantly, the ability of 2% MH to down-regulate IL-4-induced CCL26 secretion was abrogated in AHR-KO HaCaT, in keeping with the expression pattern of *CYP1A1* in the same conditions (Fig. 4a). Similar results were also obtained for *CCL26* mRNA (data not shown). Pre-treatment with AHR antagonist CH223191 abolished the MH effect on IL-4-induced CCL26 secretion ($p \leq 0.01$), again in keeping with the expression pattern of *CYP1A1* in the same conditions (Fig. 4b), and on *CCL26* mRNA expression ($p \leq 0.0001$) (Fig. 4c). Consistently, 2% MH significantly decreased IL-4-induced *CCL26* expression ($p \leq 0.01$) in NHEK and this effect was abrogated by pre-treatment with CH223191 ($p \leq 0.0001$) (Fig. 4d).

Genetically and molecularly driven impairment of the skin barrier is a key feature of AD and activation of the AHR pathway has been shown to be beneficial in restoring barrier integrity [15]. Thus, we investigated the effect of MH on the structural epidermal protein filaggrin in NHEK. MH significantly upregulated *FLG* mRNA expression ($p \leq 0.001$) in an AHR-dependent manner, as this upregulation was almost completely abrogated by CH223191 ($p \leq 0.001$) (Fig. 5a). Type 2 cytokines abundant in AD such as IL-4 are known to downregulate FLG [20]. MH significantly upregulated *FLG* expression in IL-4-treated NHEK ($p \leq 0.001$) and CH223191 significantly ($p \leq 0.001$) abrogated this effect (Fig. 5b) confirming the involvement of the AHR pathway.

4. Discussion

Here we provide evidence that MH exerts its anti-inflammatory effect in the context of type 2 skin inflammation, at least partly, by activating the AHR pathway. We show that MH significantly upregulates the expression of the AHR target gene *CYP1A1 in vitro* and *in vivo*. Moreover, this effect is abrogated *in vitro* by a specific AHR antagonist or reduced in cells partially silenced for AHR. By evaluating CYP1 enzymatic activity over time, we show that MH activate the AHR through both direct and indirect means, possibly due to the presence of both CYP1 inhibitors and AHR ligands. Importantly, MH significantly down-regulated IL-4-induced CCL26 and upregulated *FLG* expression through AHR activation, thus providing a mechanistic rationale to its anecdotical use for skin atopy [5].

Honey has recently attracted considerable interest for the treatment of allergic disorders. However, the molecular signalling pathways involved in its anti-inflammatory effect are ill-identified [6]. Most studies have focused on the inhibitory properties of specific components such as flavonoids and phenolic acids which inhibit pro-inflammatory pathways, such as nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs)[6]. Here we show that MH is able to activate the anti-inflammatory AHR pathway, which is increasingly being recognized as a key regulator of skin homeostasis [9,21]. AHR is implicated in the pathogenesis of AD [15,22] and psoriasis [12] and tapinarof, a naturally derived AHR ligand, has shown promising efficacy

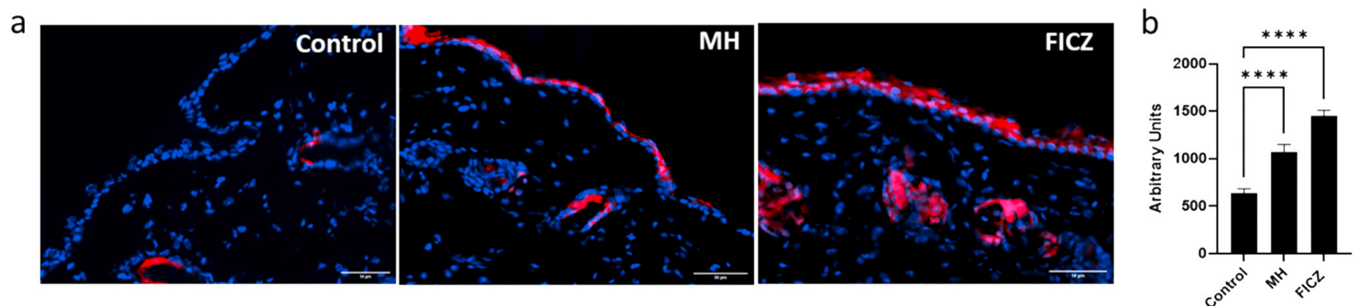

Fig. 2. a) Skin section of Cyp1a1^{Cre} x R26R^{eYFP} reporter mice topically treated with vaseline (Control), MH or FICZ for 5 days, stained for CYP1A1 (YFP, red) and DAPI (blue). Scale bar is 50 μm. Images are representative of n = 5–6 mice per group. b) Quantification of CYP1A1 expression was carried out in 3 region of interest per section (n = 15–18). One-way ANOVA and Dunnet's test for multiple comparisons. * ** * $p < 0.0001$.

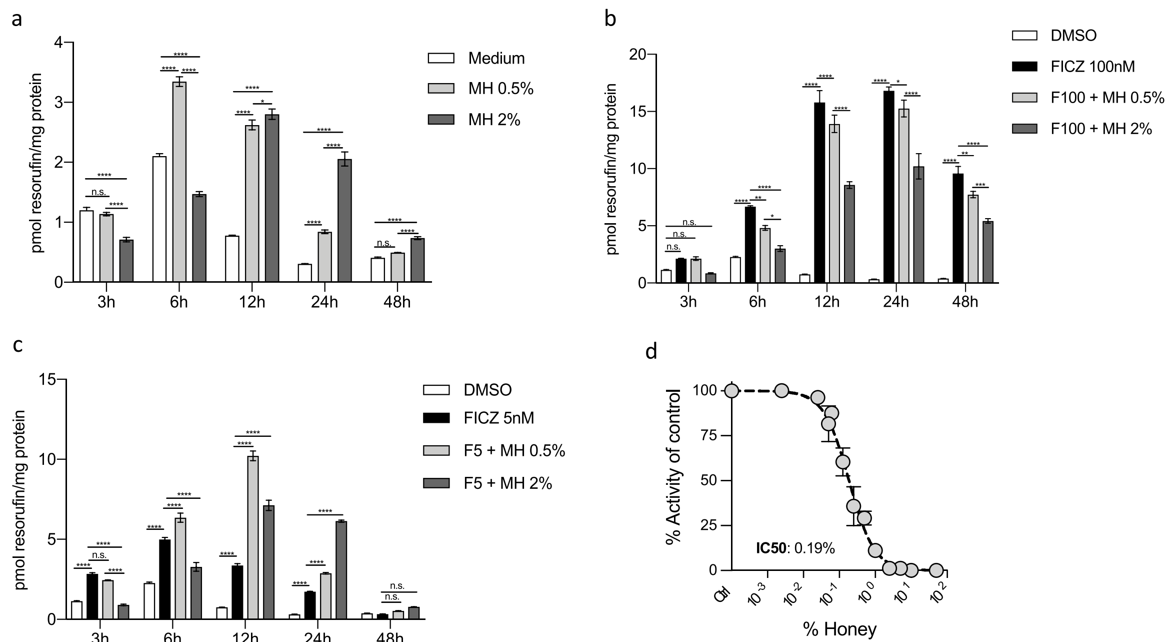

Fig. 3. a-c) CYP1 enzymatic activity measured as resorufin formation at different time points in HaCaT cells stimulated with, **a**) 0.5% and 2%, MH **b)** 100 nM FICZ with or without 0.5% and 2%, MH **c**) 5 nM FICZ with or without 0.5% and 2% MH. Concentration-effect curve of FICZ-induced CYP1 activity with equivalency of 2% and 0.2% MH in EV HaCaTs. Data shown are representative of 2 or 3 experiments and expressed as mean ± SEM. Two-way ANOVA with Sidak's multiple comparisons test. **d**) IC50 (half maximal inhibitory concentration) of serial dilutions of MH on human CYP1A1 activity. MH: Manuka honey. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$. n.s.: non-significant.

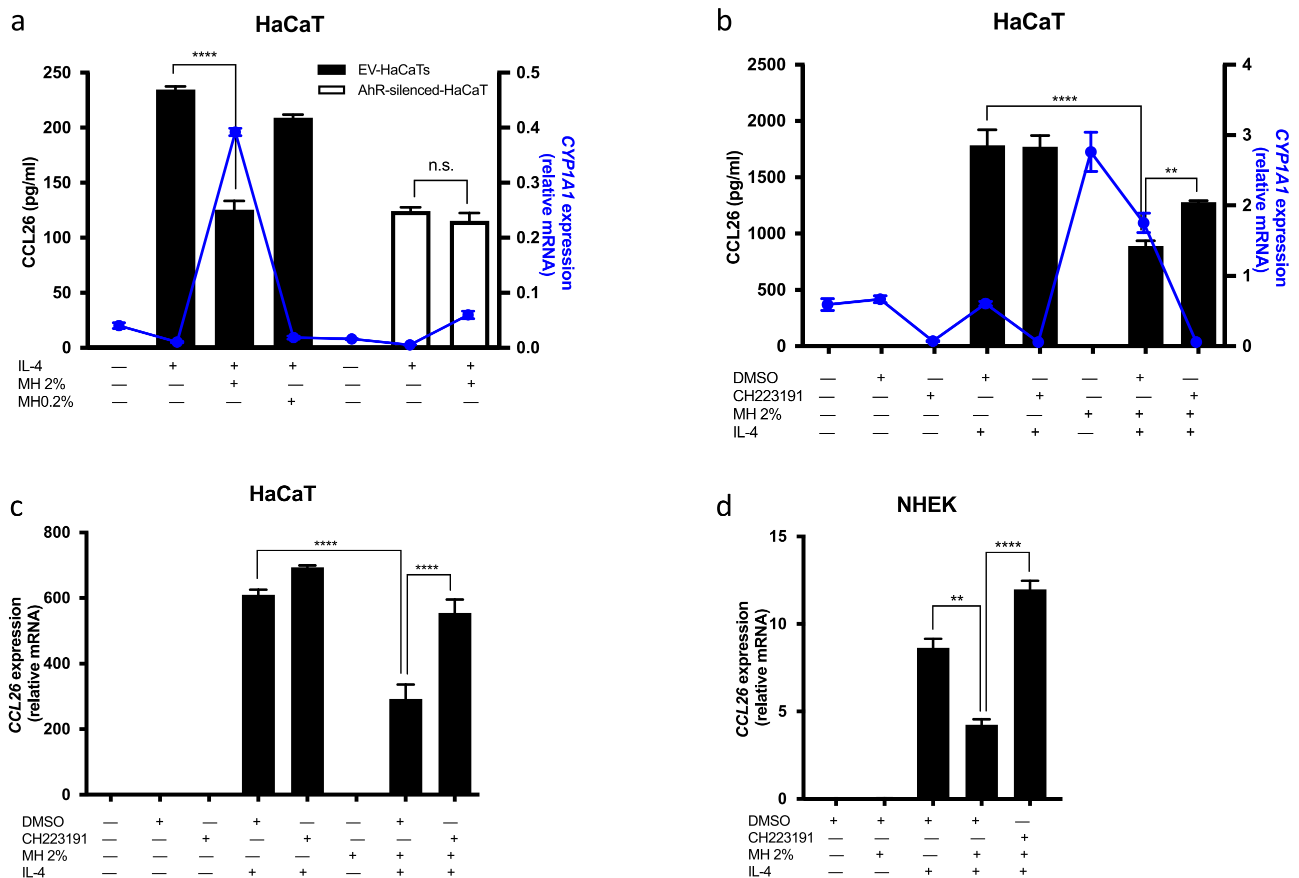

Fig. 4. a) IL-4-induced CCL26 secretion in empty vector (EV) and AHR silenced HaCaTs. The overlay graph (blue line) in (a) and (b) represent CYP*1A1* mRNA expression under the same experimental conditions. b) IL-4-induced CCL26 secretion in EV HaCaTs. c) IL-4-induced *CCL26* mRNA expression in EV HaCaTs. d) IL-4-induced *CCL26* mRNA expression in NHEK. Data shown are representative of 2 or 3 experiments and expressed as mean ± SEM. One-way ANOVA with Sidak's multiple comparisons test. * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

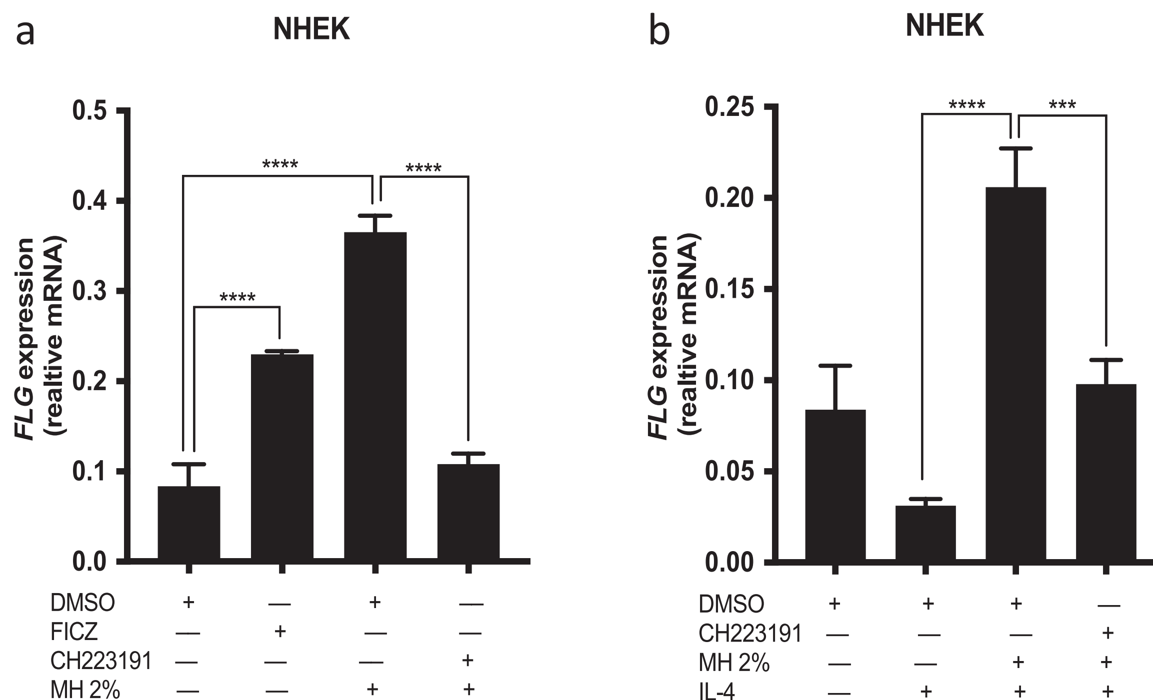

Fig. 5. a) and b) Mean mRNA expression of filaggrin (*FLG*) in NHEK. Data shown are representative of 2 or 3 experiments and expressed as mean ± SEM. One-way ANOVA with Sidak's multiple comparisons test. * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

in phase 2 clinical trials for AD [23,24] and has been approved for psoriasis by the FDA, following positive outcome in phase 3 clinical trials [25]. Honey contains several putative AHR activators, including flavonoids and indoles, such as indole-3-acetic acid (IAA) [8,9,26]. Given the very low concentration of IAA detected in 14 different Chinese raw honey samples, it appears that MH may instead contain a sizable amount of AHR activators [8,27]. This is consistent with the finding that both methanol (polar) extract and hexane (nonpolar) extract of MH down regulated IL4-induced CCL26 in HaCaTs albeit less than whole MH [4], suggesting that this effect is likely due to multiple biologically active molecules. Interestingly, unlike the prototypical AHR ligand FICZ, which induced CYP1 enzymatic activity at all tested time points, 2% MH induced CYP1 activity after 12–48 h, following an earlier inhibition at 3–6 h, although the CYP1 inhibitory effect of MH can still be observed up to 48 h when FICZ 100 nM is added to the media. Taken together, these data suggest that the observed modulation of CYP1 activity by MH at different time points is the result of the dual effect of both CYP1 inhibition, by components contained in MH such as the flavonoid quercetin [26], and direct AHR activation, by ligands in MH as well as in the culture media [10,28].

MH upregulated *FLG* expression in NHEK at baseline and in IL-4 treated NHEK in an AHR dependent manner. Heterozygous loss of function mutations in *FLG* are the strongest known genetic risk factor for atopic dermatitis [29]. In NHEK, AHR activation is a major positive regulator of *FLG* expression, which is in turn downregulated by IL-4 [30]. Compounds that upregulate *FLG* could attenuate the development of atopic dermatitis and ameliorate its manifestations [31]. In addition, *FLG* expression inhibits staphylococcal α-toxin-mediated keratinocyte death [32]. Thus, activation of AHR *via* MH has the potential to attenuate allergic skin inflammation and restore skin barrier integrity. Further *in vivo* studies using animal models of AD are needed to fully explore and dissect the beneficial effect of MH in atopic skin inflammation. Other properties of MH such as its antioxidant [33] and anti-staphylococcal [34] effects may also contribute to its action in AD, independently of the AHR pathway.

Our study is limited by the lack of identification of all the specific compounds that act as AHR ligands and those that are CYP1a1 inhibitors in MH. This should be investigated as another major project. Also, testing the effect of MH in an AD mouse model and in human subjects should be performed as another project to further confirm our findings. In addition, the mechanism by which AHR activation attenuates IL4-induced CCL26 expression is yet to be defined. We have previously shown that treatment of HaCaT cells with MH does not inhibit STAT-6 phosphorylation [4] thus suggesting that the AHR-mediated effect may be independent of STAT6. It is also possible that ligated AHR may inhibits phosphorylated STAT-6 DNA binding, either directly or indirectly, and this is the subject of an ongoing investigation by our group.

Activation of the AHR pathway by MH offers a novel intriguing and at least a partial explanation of its anecdotical use in atopic dermatitis. Beyond the anti-allergic effect, AHR signaling attenuates psoriatic skin inflammation *in vivo* and *ex vivo* by reducing the responsiveness of keratinocytes to inflammatory stimuli [12]. On the other hand, systemic AHR inhibition in humans, a side effect of the protein kinase inhibitor vemurafenib, leads to the development of generalized cutaneous maculopapular rash and a pro-inflammatory state [35]. Thus, the AHR-activating properties of MH could potentially have broader clinical implications in a range of skin inflammatory conditions.

Funding source

This study was funded by the Deanship of Scientific Research at King Saud University through the research group project No. RGP-190 (to A. A.A), a National Psoriasis Foundation Translational Award 814364 (to P. D.M.), the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' National Health Service (NHS) Foundation Trust and King's College London (to J.B. and P.D.M), the Medical Research Council/British Association of Dermatologists/ British Skin Foundation for a Clinical Research Fellowship No. MR/ P001394/1 (to W.A), the Swedish Research Council (VR) grant No. 2020-03418 (to E.W), and by a Wellcome Trust Grant 210556/Z/18/Z (to B.S). . The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health, or other funding bodies. For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted

Manuscript version arising from this submission.

CRediT authorship contribution statement

Abdullah A. Alangari: Conceptualization, Formal analysis, Investigation, Writing – original draft, Funding acquisition. **Matin D. Ashoori:** Investigation, Formal analysis: **Wisam Alwan:** Methodology, Investigation. **Hannah R. Dawe:** Formal analysis, Visualization; **Brigitta Stockinger:** Resources; **Jonathan N. Barker:** Resources, Funding acquisition; **Emma Wincent:** Formal analysis, Investigation, Methodology, Writing – review & editing, Funding acquisition; **Paola Di Meglio**: Supervision; Funding acquisition, Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on online repositories after publication.

Acknowledgements

The authors would like to thank Dr. V. Suen for isolating human primary keratinocytes, H. Sreeneebus for collection of clinical samples and M. Tolaini for helping with animal experiments. We would like to acknowledge the Biological Research Facility at the Francis Crick Institute for expert breeding and maintenance of mice and Biological Services at King's College London for animal husbandry.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106848.

References

[1] D.S. Lee, S. Sinno, A. Khachemoune, Honey and wound healing: an overview, Am. J. Clin. Dermatol. 12 (3) (2011) 181–190.
[2] J. Majtan, Honey: an immunomodulator in wound healing, Wound Repair Regen. 22 (2) (2014) 187–192.
[3] S. El-Ashram, L.M. El-Samad, A.A. Basha, A. El Wakil, Naturally-derived targeted therapy for wound healing: beyond classical strategies, Pharm. Res 170 (2021), 105749.
[4] A.A. Alangari, K. Morris, B.A. Lwaleed, L. Lau, K. Jones, R. Cooper, et al., Honey is potentially effective in the treatment of atopic dermatitis: clinical and mechanistic studies, Immun. Inflamm. Dis. 5 (2) (2017) 190–199.
[5] P. McLoone, A. Oluwadun, M. Warnock, L. Fyfe, Honey: a therapeutic agent for disorders of the skin, Cent. Asian J. Glob. Health 5 (1) (2016) 241.
[6] P.Y.A. Yong, F. Islam, H. Hazeera, D.A. Israf, J.W. Tan, C.L. Tham, The potential use of honey as a remedy for allergic diseases: a mini review, Front. Pharmacol. (2020).
[7] J.M. Alvarez-Suarez, M. Gasparrini, T.Y. Forbes-Hernandez, L. Mazzoni, F. Giampieri, The composition and biological activity of honey: a focus on manuka honey, Foods 3 (3) (2014) 420–432.
[8] Q. Wang, W.J. Cai, L. Yu, J. Ding, Y.Q. Feng, Comprehensive profiling of phytohormones in honey by sequential liquid-liquid extraction coupled with liquid chromatography-mass spectrometry, J. Agric. Food Chem. 65 (3) (2017) 575–585.
[9] B. Stockinger, P. Di Meglio, M. Gialitakis, J.H. Duarte, The aryl hydrocarbon receptor: multitasking in the immune system, Annu Rev. Immunol. 32 (2014) 403–432.
[10] E. Wincent, J. Bengtsson, A. Mohammadi Bardbori, T. Alsberg, S. Luecke, U. Rannug, et al., Inhibition of cytochrome P4501-dependent clearance of the endogenous agonist FICZ as a mechanism for activation of the aryl hydrocarbon receptor, Proc. Natl. Acad. Sci. USA 109 (12) (2012) 4479–4484.
[11] J. Yu, Y. Luo, Z. Zhu, Y. Zhou, L. Sun, J. Gao, et al., A tryptophan metabolite of the skin microbiota attenuates inflammation in patients with atopic dermatitis through the aryl hydrocarbon receptor, J. Allergy Clin. Immunol. 143 (6) (2019), 2108-19 e12.
[12] P. Di Meglio, J.H. Duarte, H. Ahlfors, N.D. Owens, Y. Li, F. Villanova, et al., Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions, Immunity 40 (6) (2014) 989–1001.
[13] C. Shi, H. Zhang, X. Wang, B. Jin, Q. Jia, Y. Li, et al., Cinnamtannin D1 attenuates autoimmune arthritis by regulating the balance of Th17 and treg cells through inhibition of aryl hydrocarbon receptor expression, Pharm. Res 151 (2020), 104513.
[14] W. Jing, S. Dong, X. Luo, J. Liu, B. Wei, W. Du, et al., Berberine improves colitis by triggering AhR activation by microbial tryptophan catabolites, Pharm. Res 164 (2021), 105358.
[15] E.H. van den Bogaard, J.G. Bergboer, M. Vonk-Bergers, I.M. van Vlijmen-Willems, S.V. Hato, P.G. van der Valk, et al., Coal tar induces AHR-dependent skin barrier repair in atopic dermatitis, J. Clin. Invest 123 (2) (2013) 917–927.
[16] C. Schiering, E. Wincent, A. Metidji, A. Iseppon, Y. Li, A.J. Potocnik, et al., Feedback control of AHR signalling regulates intestinal immunity, Nature 542 (7640) (2017) 242–245.
[17] M. Kyoreva, Y. Li, M. Hoosenally, J. Hardman-Smart, K. Morrison, I. Tosi, et al., CYP1A1 enzymatic activity influences skin inflammation via regulation of the AHR pathway, J. Invest Dermatol. 141 (6) (2021), 1553-1563 e3.
[18] E. Fritsche, C. Schafer, C. Calles, T. Bernsmann, T. Bernshausen, M. Wurm, et al., Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmatic target for ultraviolet B radiation, Proc. Natl. Acad. Sci. USA 104 (21) (2007) 8851–8856.
[19] U. Laggner, P. Di Meglio, G.K. Perera, C. Hundhausen, K.E. Lacy, N. Ali, et al., Identification of a novel proinflammatory human skin-homing Vgamma9Vdelta2 T cell subset with a potential role in psoriasis, J. Immunol. 187 (5) (2011) 2783–2793.
[20] M.D. Howell, B.E. Kim, P. Gao, A.V. Grant, M. Boguniewicz, A. Debenedetto, et al., Cytokine modulation of atopic dermatitis filaggrin skin expression, J. Allergy Clin. Immunol. 120 (1) (2007) 150–155.
[21] M. Furue, A. Hashimoto-Hachiya, G. Tsuji, Aryl hydrocarbon receptor in atopic dermatitis and psoriasis, Int J. Mol. Sci. 20 (21) (2019).
[22] J.P.H. Smits, T.H.A. Ederveen, G. Rikken, N.J.M. van den Brink, I. van Vlijmen-Willems, J. Boekhorst, et al., Targeting the cutaneous microbiota in atopic dermatitis by coal tar via AHR-dependent induction of antimicrobial peptides, J. Invest Dermatol. 140 (2) (2020), 415-24 e10.
[23] J. Peppers, A.S. Paller, T. Maeda-Chubachi, S. Wu, K. Robbins, K. Gallagher, et al., A phase 2, randomized dose-finding study of tapinarof (GSK2894512 cream) for the treatment of atopic dermatitis, J. Am. Acad. Dermatol. 80 (1) (2019), 89-98 e3.
[24] A.S. Paller, L.S. Gold, J. Soung, A.M. Tallman, D.S. Rubenstein, M. Gooderham, Efficacy and patient-reported outcomes from a Phase IIb, randomized clinical trial of tapinarof cream for the treatment of adolescents and adults with atopic dermatitis, J. Am. Acad. Dermatol. (2020).
[25] M.G. Lebwohl, L. Stein Gold, B. Strober, K.A. Papp, A.W. Armstrong, J. Bagel, et al., Phase 3 trials of tapinarof cream for plaque psoriasis, N. Engl. J. Med 385 (24) (2021) 2219–2229.
[26] C.W. Chan, B.J. Deadman, M. Manley-Harris, A.L. Wilkins, D.G. Alber, E. Harry, Analysis of the flavonoid component of bioactive New Zealand manuka (Leptospermum scoparium) honey and the isolation, characterisation and synthesis of an unusual pyrrole, Food Chem. 141 (3) (2013) 1772–1781.
[27] S. Heath-Pagliuso, W.J. Rogers, K. Tullis, S.D. Seidel, P.H. Cenijn, A. Brouwer, et al., Activation of the Ah receptor by tryptophan and tryptophan metabolites, Biochemistry 37 (33) (1998) 11508–11515.
[28] A. Mohammadi-Bardbori, J. Bengtsson, U. Rannug, A. Rannug, E. Wincent, Quercetin, resveratrol, and curcumin are indirect activators of the aryl hydrocarbon receptor (AHR), Chem. Res Toxicol. 25 (9) (2012) 1878–1884.
[29] A.D. Irvine, W.H. McLean, D.Y. Leung, Filaggrin mutations associated with skin and allergic diseases, N. Engl. J. Med 365 (14) (2011) 1315–1327.
[30] M. Furue, G. Tsuji, C. Mitoma, T. Nakahara, T. Chiba, S. Morino-Koga, et al., Gene regulation of filaggrin and other skin barrier proteins via aryl hydrocarbon receptor, J. Dermatol. Sci. 80 (2) (2015) 83–88.
[31] A. Otsuka, H. Doi, G. Egawa, A. Maekawa, T. Fujita, S. Nakamizo, et al., Possible new therapeutic strategy to regulate atopic dermatitis through upregulating filaggrin expression, J. Allergy Clin. Immunol. 133 (1) (2014), 139-46 e1-10.
[32] A.M. Brauweiler, L. Bin, B.E. Kim, M.K. Oyoshi, R.S. Geha, E. Goleva, et al., Filaggrin-dependent secretion of sphingomyelinase protects against staphylococcal alpha-toxin-induced keratinocyte death, J. Allergy Clin. Immunol. 131 (2) (2013), 421-7 e1-2.
[33] M. Hunter, R. Ghildyal, N.M. D'Cunha, C. Gouws, E.N. Georgousopoulou, N. Naumovski, The bioactive, antioxidant, antibacterial, and physicochemical properties of a range of commercially available Australian honeys, Curr. Res Food Sci. 4 (2021) 532–542.
[34] D.A. Carter, S.E. Blair, N.N. Cokcetin, D. Bouzo, P. Brooks, R. Schothauer, et al., Therapeutic manuka honey: no longer so alternative, Front Microbiol 7 (2016) 569.
[35] H.C. Hawerkamp, A. Kislat, P.A. Gerber, M. Pollet, K.M. Rolfes, A.A. Soshilov, et al., Vemurafenib acts as an aryl hydrocarbon receptor antagonist: Implications for inflammatory cutaneous adverse events, Allergy (2019).