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A Rabbit Model for Assessment of Volatile Metabolite Changes Observed from Skin: a Pressure Ulcer Case Study

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Abstract

Human skin presents a large, easily accessible matrix that is potentially useful for diagnostic applications based on whole body metabolite changes – some of which will be volatile and detected using minimally invasive tools. Unfortunately, identifying skin biomarkers that can be reliably linked to a particular condition is challenging due to a large variability of genetics, dietary intake, environmental exposures within human populations. This leads to a paucity of clinically validated volatile skin biomarker compounds. Animal models present a very convenient and attractive way to circumvent many of the variability issues. The rabbit (*Leporidae*) is a potentially logistically useful model to study the skin metabolome, but very limited knowledge of its skin metabolites exists. Here we present the first comprehensive assessment of the volatile fraction of rabbit skin metabolites using polydimethylsiloxane sorbent patch sampling in conjunction with gas chromatography / mass spectrometry (GC/MS). A collection of compounds that are secreted from rabbit skin was documented, and predominantly acyclic long-chain alkyls and alcohols were detected. We then utilized this animal model to study differences between intact skin and skin with early pressure ulcers, as the latter are a major problem in intensive care units. Four New Zealand female white rabbits underwent ulcer formation on one ear with the other ear as a control. Early-stage ulcers were created with neodymium magnets. Histologic analysis showed acute heterophilic dermatitis, edema, and micro-hemorrhage on the ulcerated ears with normal findings on the control ears. The metabolomic analysis revealed subtle but noticeable differences, with several compounds associated with the oxidative stress-related degradation of lipids found to be present in greater abundances in ulcerated ears. The metabolomic findings correlate with histologic evidence

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of early-stage ulcers. We postulate that the *Leporidae* model recapitulated the vascular changes associated with ulcer formation. This study illustrates the potential usefulness of the *Leporidae* model for skin metabolome studies as illustrated by this study of early-stage ulcer formation.

Keywords

skin; volatile organic compounds (VOCs); pressure ulcers; gas chromatography / mass spectrometry (GC/MS); Leporidae

1. Introduction

Humans and animals produce metabolite compounds which may be used in noninvasive disease detection (1–8). In particular, skin metabolites can be measured and analyzed (9), and some of these compounds may be useful as biomarkers for disease. Skin metabolite analysis has been performed on human subjects with chronic arterial ulcers using gas chromatography / mass spectrometry (GC/MS) (10); although to our knowledge, there are a limited number of studies assessing the general skin metabolome, especially the volatile fraction. Although a comprehensive study of the non-volatile metabolome along with corresponding bacteriome on human skin has been conducted (11), the research is still at its nascent stage. In particular, the major impediments to identifying specific skin-borne biomarkers of particular conditions in human studies are: population genetic variability, differences in diet and life style choices such as smoking or the use of skin products. To account for these factors, large participant cohorts are required to assemble sufficient statistics to discern specific factors contributing to the chemical makeup of skin. Both biological and environmental variability can obfuscate potential metabolite differences for specific conditions. Human population studies can be expensive, require specialized facilities and carry heavy regulatory burdens. As a result, currently there are no clinically validated skin biomarker compounds that may be used for commercial diagnostic purposes.

Animal models present a convenient and attractive way to circumvent some of these issues, as it is often possible to select animals with a specific lineage and rigorously control external factors. Furthermore, it is often possible to induce a specific condition in an animal so it can serve as its own control, thus further reducing variability. The rabbit (*Leporidae*) model is potentially a very useful model to study the skin metabolome. The animals are sufficiently large with easily accessible ear lobes, which makes logistics of skin metabolome collection far easier than in smaller animals, specifically *Murinae*. Another advantage of the *Leporidae* model is the potential to isolate the vascular component of skin metabolite production. A lack of sebaceous glands significantly reduces the complexity of the resultant metabolome compared to human skin. Finally, animals with minimal genome variations as well as animals with specific genetic differences are commercially available, thus further aiding more rigorous control of experimental studies. Unfortunately, current knowledge of the *Leporidae* volatile skin metabolome is very limited. A comprehensive assessment of the baseline chemical composition is essential before any potential practical use of such model. In this work, we present a first comprehensive study of the volatile fraction of the skin metabolome for the rabbit model (*Leporidae*).

As a proof-of-concept, we performed a sub-study to assess if skin metabolites change as a result of experimentally-produced pressure ulcers using the rabbit model. Pressure ulcers are a major problem affecting hospitalized patients and residents of long-term care facilities. Economic estimates cite an annual expenditure of \$2.5M per year in care directly related to pressure ulcers (12) and \$11B in direct and indirect costs from 1993–2006 (13). Over 50% of patients admitted to an intensive care unit at a single institution developed stage I or greater pressure ulcers (14), and another study identified that neonates in pediatric intensive care units are more susceptible to device-related pressure ulcers than previously thought (15). Russo et al. identified that patients who develop pressure ulcers in a hospital are more likely to be sent to a long-term care facility where they face an increased risk of death (13), largely due to infections (16). Given this burden, major efforts exist to prevent, recognize, and treat pressure ulcers (17).

Two useful clinical tools to identify patients at risk for pressure ulcers are the Norton (18) and Braden (19) scales. They identify risk based on a variety of clinical factors including baseline mobility, activity level, nutritional status, and others. Although the tools are valid, their sensitivities range from 70–90%, with specificities from 60–80% (20), and their predictive values are moderate at best (21). Moreover, the tools may not be better at predicting ulcer development than clinical judgment alone (21). Given the enormous economic, functional, and social impact of pressure ulcers on affected patients, improvements in screening would have a sizeable effect. The overarching aim of this study is to lay a foundation for an accessible animal model to study skin biomarkers which may ultimately be a platform to study human skin diseases such as pressure ulcers.

2. Materials and Methods

2.1 Animals

Four 8-week old New Zealand White female rabbits (*Oryctolagus cuniculus*) were obtained from Charles River Laboratories (Wilmington, MA). The rabbits were fed standard chow, and housed in separate AAALAC-certified metal units with daily bedding changes. Food and water were provided ad libitum throughout the study. All experiments on the rabbits were performed at 32 weeks of age when the ear thickness was approximately 3.7 mm. Each of the four rabbits underwent three experiments and all metabolomic data were pooled. Rabbits were assessed for health by the laboratory manager and staff veterinarian on a regular basis per the research unit protocol in adherence with AAALAC standards. This experiment was approved by the Animal Use and Care Administrative Advisory Committee at University of California, Davis (IACUC protocol #17749).

2.2 Metabolite Sampling

The rabbits were acclimated to an Elizabethan collar to prevent scratching their ears during the sampling period. Individual rabbits were removed from their cage and manually secured. The hair on the ventral ear surfaces was trimmed to <1 mm length one day prior to treatment. The skin metabolites were sampled using custom made polydimethylsiloxane (PDMS) patches (Goodfellow, Coraopolis, PA) following a procedure similar to that described earlier for humans (22, 23). The 0.6 mm thick material was cut into 5 mm

diameter round patches using a standard hole punch. One side of each patch was labelled as the reverse side, the other being the sampling side held against the skin. The patches were placed into borosilicate vials and moved into a vacuum oven set at 160 °C to remove any adsorbed chemicals; this baking step was conducted for 12 h. The temperature and duration of the baking step were necessary to minimize background contaminants present in PDMS patches as determined in prior experiments. After the baking period, the vials with PDMS were removed from the vacuum oven, purged with ultra-high purity nitrogen, and tightly capped with stainless steel screw caps with a 35 (Shore A) PTFE/silicone septum.

For the sampling, the ventral surface of the right ear was cleaned with 70% ethanol solution and allowed to air dry. Six PDMS patches were placed over the right ear (Figure 1). These PDMS patches were covered with a protective and inert polytetrafluoroethylene (PTFE) patch and affixed with Tegaderm® dressings. Skin metabolites were sampled with the PDMS for 30 min, and patches were removed, placed into separate nitrogen-purged borosilicate vials, capped, and routed for gas chromatography-mass spectrometry (GC/MS) analysis. The optimal sampling methodology was determined in a separate set of preliminary experiments (data not shown). A set of control samples (two each) were collected along with the skin samples: room air blanks where PDMS patches were exposed to vivarium air for 30 min; cage blanks where PDMS patches were exposed to animal cage air for 30 min; sampling blanks where PDMS patches were covered by PTFE patches and Tegaderm dressing for 30 min; and control blanks which were clean PDMS patches. All four rabbits underwent three separate and identical experiments.

2.3 GC/MS Analysis

For the GC/MS analysis, a Varian 3800 GC (VF-5ms 5% phenol/95% 30 m, 0.25 mm, 0.25 µm PDMS column; Varian, Walnut Creek, CA), 4000 Ion Trap MS (Varian) equipped with Electron Ionization source (EI) was employed. The vials containing PDMS patches with the sampling side up were placed on the instrument tray. For analysis, each vial was transferred by auto-sampling robot arm from the tray into heater/agitator station set at 200 °C. The vial was heated for 15 min with agitation to displace PDMS patch during desorption. After 15 min, the desorbed volatiles released into the headspace were sampled using an automatic headspace injection syringe. The syringe needle was inserted through the cap septum and 500 µL of headspace was inspired. Upon sampling, the syringe content was injected into the GC inlet, and the syringe was purged with helium carrier gas for 30 sec to prevent carryover for the next injection. The syringe temperature was maintained at 80 °C during sampling to avoid carryover. The ion trap was set to 250 °C, the MS set to 100 °C, and the transfer line set to 250 °C.

The sampled chemicals were then analyzed using a GC protocol specifically developed for optimal separation of skin metabolites. The GC protocol was set as follows: cryofocusing on the head of the column at -10 °C for 1 min; 50 °C/min oven ramp to 40 °C, 20 °C/min oven ramp to 100 °C, 5 °C/min oven ramp to 180 °C, 10 °C/min oven ramp to 250 °C, 20 /min oven ramp to 280 °C, and a 5 min hold to purge the column for a complete run time of 34.5 min. The helium carrier gas was set to constant 1 mL/min flow. The m/z range scanned was 35–250 Th. In order to eliminate potential systematic bias, the samples were randomized

during analysis. Empty vial blanks and collected experimental blanks were randomly interspersed with the samples. Collected GC/MS data were then analyzed offline. Our indirect sampling method does not allow for introduction of a standard during the sampling stage; however, the instrument performance and day-to-day reproducibility were controlled by injecting a standard Grob DA 280 Column Test Mix (Restek, Bellefonte, PA).

For chemical identification, the mass spectra were matched using NIST Mass Spectral Search Software v. 2.0 with NIST 2005 and Wiley 2009 MS libraries as described previously (25). If necessary, deconvolution of mass spectra was carried out with the AMDIS software. Background peaks, external contaminants (e.g. phthalates) and peaks with unacceptably low S/N ratio were excluded. The most likely candidate compound(s) were suggested as a tentative identification whenever appropriate (high match probability, typically over 80%, high forward and reverse scores, typically over 700 each). If no conclusive identification were possible, but reasonable assumption about the structure could be made, a tentative compound class was suggested.

2.4 Experimental Pressure Ulcers

A 7/16-in diameter x 1/16-in thick neodymium magnet (KJ Magnetic Inc, Pipersville, PA) with attached string loop was placed on the left ear ventral surface and a second identical magnet was attached to the dorsal side to complete a magnetic seal (Figure 2). These were placed in the area between the central artery and marginal veins.

The magnets were calculated to produce a force of 48.66 mmHg/cm² (for an average ear thickness of 3.7 mm) based on the following experiments: magnets of varying sizes were placed on both sides of a 3.7 mm thick group of rubber bands. The force required to pull the magnets apart was measured using a hanging spring scale (Cole Parmer, Vernon Hills, IL). We selected the 7/16-inch diameter x 1/16-in thick magnet based on its convenient size and its force at 3.7 mm separation, which is close to the force used previously to induce early-stage ulcer formation in rabbit ears (24).

The magnets were kept on the right ear for 90 min before being removed. Early-stage ulcers were created and assessed based on the following criteria: blanched skin immediately (Figure 3) followed by nonblanching erythema, no obvious skin breakdown or necrosis, gross resolution of the erythema within two hours, and no recurrence of the ulcer after three hours with preservation of grossly viable tissue. Metabolite sampling was performed as above in section 2.2.

2.5 Histologic Analysis

Two rabbits were euthanized for histologic analysis of both the ulcerated and non-ulcerated ears approximately 1 hour after experimental ulcer formation. The ears were removed and sections from the ulcer and corresponding area of the control ear were fixed and sections made. Gross inspection as well as hemotoxylin and eosin staining was performed by a pathologist. Inflammatory cells were grossly described.

2.6 Statistical Analysis

For all of the samples, a comprehensive table of all peaks in the chromatogram was compiled. Following this, a deconvolution method using AMDIS (Automated Mass spectral Deconvolution & Identification Software, v 2.64) was applied to remove background noise and overlapping peaks. Peak alignment was then performed using Mass Profiler Professional (Agilent, Santa Clara, CA). Chemicals for each group (ulcer and control) were pooled. These chemicals were derived from three technical replicates per sample, and three experimental replicates. The presence of a chemical in >70% or <20% of the pooled metabolic samples for each group (ulcer or control) was required to consider the chemical biologically reproducible and potentially relevant. Peaks which did not satisfy these requirements were discarded. Also, peaks present in <20% of one group and <20% of the other group were also excluded. Peaks corresponding to PDMS/column/septa bleed peaks (siloxanes) and environmental contaminants (e.g. phthalates) were excluded. All of the peaks present in blanks were also excluded. For statistical comparisons of chemical abundances between groups, we used unpaired *Student's* t-testing with an $\alpha=0.05$ and $p<0.05$ considered significant.

3. Results

In this work, we have demonstrated that it is possible to detect a significant number of skin metabolites for the *Leporidae* model system. These compounds are easily sampled and analyzed, and they represent several compound classes. An example chromatogram shows the richness of metabolite content observed from healthy skin using the described sampling procedures (Figure 4), including many small lower abundance biogenic compounds that close to the noise floor (Figure 4, inset). There were approximately 100–150 distinct compounds indexed across multiple animals. The summary of indexed compounds is provided (Table A1, Appendix). The identified compounds categorized according to functional group are also provided (Table 1). The majority of the detected metabolites are aliphatic compounds such as alkanes and alcohols.

Some of the detected metabolites are volatile organic compounds (VOCs), but a great number of long-chain waxy compounds were also detected. These metabolites are expected to be present on skin, and a number of the detected metabolites have been reported coming from human skin and body fluids (26).

Using the neodymium magnets enabled the formation of early-stage pressure ulcers as defined above. No rabbits showed signs of more advanced ulcers which would include gross skin breakdown, bleeding, skin necrosis, or skin perforation. Histologic analyses of two rabbits showed that although there were no gross lesions, there were 1–1.5 cm erythematous lesions with acute heterophilic dermatitis, edema, and micro-hemorrhage on the ulcerated ears. The contralateral control ears had normal findings.

The compounds detected from ulcerated ears closely matched the compounds detected in control ears (Table A1, Appendix). At the same time, there were 12 biologically-relevant compounds that varied between the ulcer and control groups (Table 2). As per Section 2.6 *Statistical Analysis*, these twelve compounds were identified in >70% of the pooled samples

from one group versus <20% of the pooled samples from the other group. After comparing the abundances of these compounds with an unpaired *Student's t-test*, one compound was statistically significantly more abundant in the ulcer group and was minimally present in the control group. The ulcer-specific compound was tentatively identified as an 8-carbon branched alcohol.

4. Discussion

This paper describes a first comprehensive study of the volatile skin metabolome of rabbits with the intent of laying a foundation for the use of the *Leporidae* model for skin metabolite measurements. In this work, we have reported a number of important findings. First, the rabbit model is relatively simple to use and clearly demonstrates an abundance of skin metabolites that are discretely different from background, non-skin metabolites. Approximately 150 unique compounds have been indexed. As the largest external organ, skin is subject to environmental contamination with organic and inorganic products. Here we were able to show that rabbits produce skin metabolites that reflect both the external, non-skin environment, and skin-borne metabolome. This finding indicates that skin metabolite analysis may be useful to monitor animals, and eventually humans, independent of environment. Given that animal models are cheaper and simpler to study than human models, this *Leporidae* system has the potential as a platform to study skin emanations over time in healthy and disease states.

The skin metabolomic products observed here are mainly waxy compounds that may be derived from the oxidative breakdown of lipid moieties, all expected compounds that might derive from skin surface lipids as one of several potential sources. Some of the detected metabolites are volatile organic compounds (VOCs), but a great number of long-chain waxy compounds were also detected. The employed analytical methodology of headspace sampling and GC/MS analysis allows for detection of both volatile and semi-volatile compounds. The non-volatile compounds could be detected due to physical contact of the sampling PDMS patch with the skin and then compounds volatilized by the high temperature at the desorption step. As can be seen from Tables 1 and A1, the majority of the detected metabolites are aliphatic compounds such as alkanes and alcohols, both normal and branched. Due to the paucity of data on volatile metabolome for animal models, the most appropriate comparison could be made to human skin. The detected metabolites are expected to be present on skin, and a number of the detected metabolites have been reported coming from skin as well as humans body fluids (26).

Some compounds identified in our study, such as benzyl benzoate (Table A1), are likely of exogenous origin. This compound is a natural component of cinnamon essential oil as well as a common fragrance ingredient, artificial flavor, and preservative. Other compounds such as triacetin (used as a cosmetic biocide, plasticizer, and solvent in cosmetic formulations) or terpenes are likely also originate from the environment. In this regard, the skin metabolome may be a useful source of information regarding a human or animal's environment and exposures. The *Leporidae* model controls the animal's environment and provides ease of sampling compared to the murine model, where skin sampling is much less convenient. However, the inherent complexity of obtaining pure biologic samples makes it possible that

exogenous contaminants may be present on rabbit skin and not absorbed by the room air and cage blanks. This underscores the importance assessing each compound for biologic relevance.

In contrast, other compounds we identified are likely biogenic and could be endogenously produced or metabolized. For example, diethyl succinate occurs naturally in both plant and animal tissues and is most likely of endogenous origin. This compound is present in the citric acid cycle and is a by-product of the fermentation of sugar. It, therefore, likely originates either from animal feed or may be excreted through the skin, and thus may be possibly linked to internal metabolic processes. Several examples of other compounds that may not be skin-borne but are clearly of biological origin can be seen in Tables A1 and 1. Some of these compounds may be indicative of systemic or local metabolic process, but not necessarily skin-related.

Skin is a complex organ consisting of several tissue layers including epidermis, dermis, hypodermis, blood vessels, lymphatics, connective tissue, fat, and smooth muscle. To date, the tissue levels of volatile and semi-volatile metabolite emanations are not well-described with respect to skin. In truth, skin metabolite emanations likely originate from several tissue types and blood vessels. Consequently, it is plausible that skin metabolites may reflect both the local skin environment and systemic health. This *Leporidae* model may be useful to study the local skin environment, which we believe is indicated due to the detection of breakdown products of skin lipids. These products are similar to those seen in at least one human model of disease (10), which supports a *Leporidae* model as a reflection of the outer layers of human skin (epidermis and possibly dermis). Future experimentation with this model may reveal that systemic manipulations may be seen using skin metabolite analysis. Indeed, a strength of this model is its ability to isolate contributions of the epidermis and dermis to the skin metabolome.

This *Leporidae* model has the potential to serve as a platform to study skin disease states. As a proof-of-concept, we induced experimental and early-stage pressure ulcers on the rabbit ears. At least one chemical was significantly elevated when measured from the ears with ulcers compared to the intact ears, tentatively identified as an 8-carbon alcohol (see Table 2). The conclusive identification for this compound is not possible due to insufficient signal-to-noise. Several other compounds had a trend towards elevated levels in ulcerated ears compared to the healthy ones, although the statistical power of the results is not sufficient to make definitive conclusions. All of these compounds are of likely biogenic origin and could be expected to be present on skin. Interestingly, these compounds, especially branched hydrocarbons and alcohols have been previously identified as the oxidative stress products that result from oxidative radical-driven degradation of lipids (27–31). One of the compounds, 1,3-bis(1,1-dimethylethyl)benzene, was reported as a marker of irradiation in beef (32) and other gamma-irradiated food (33). This compound is formed through the reaction of methyl radicals, produced by irradiation with aromatic compounds such as xylenes.

It is reasonable to suggest that a majority of the compounds from Table 2 that are indicative of ulcer formation are associated with lipid degradation, possibly via non-specific oxidation

induced by oxidative stress. It is established that induction of pressure ulcers is concomitant with oxidative damage, and anti-oxidant defense helps to alleviate pressure sores such as those that occur in the setting of diabetes (34–38). Our findings are consistent with the premise of an oxidative environment in ulcerated tissue. Though these findings are not robust, they do indicate that we were able to identify a metabolomic difference from tissues undergoing a complex process: ulcer formation. The process of pressure formation involves vascular compression, tissue hypoperfusion, skin cell necrosis, and, possibly, reperfusion injuries once the pressure is relieved (39). Occasionally infection complicates pressure ulcers (40). Clearly, there exists a spectrum of injury as noted by several clinical pressure ulcer grading scales such as the Braden Scale and others (19).

Our aim in the sub-study was to note if we could detect early-stage ulcer by metabolomics analysis. An advantage of the *Leporidae* model is the isolation of a vascular component of ulcer formation. Since the rabbit ears are highly vascularized and vascular occlusion/reperfusion was easily seen, we interpreted that the metabolites we identified originated from vascular changes associated with ulcer formation. However, this conclusion requires further validation and it is likely that pressure ulcer formation on human skin is far more complex. Nevertheless, detection of metabolites that originate from a vascular source in pressure ulcer formation may be important for establishing mechanistic details of human pressure ulcers. Other aspects need to be highlighted. First, the ears with early pressure ulcer formation underwent apparent morphologic changes. They blanched (vascular compression) and then became hyperemic (vascular reperfusion); although the skin surface was grossly intact, there were obvious vascular changes. The finding of a unique compound from the skin surface could indicate that it derived from the skin tissue surface or from a vascular reperfusion phenomenon, though this investigation precluded identifying an exact source. The identified unique 8-carbon branched alcohol is commonly occurring. Therefore, we have refrained from postulating the compound origin. We also speculate that the outer layers of skin, the epidermis and dermis in particular, may prevent many metabolites from reaching the skin surface for analysis. In addition, our technique and animal model appears to gather metabolites mainly from the skin surface. However, some metabolites differences are seen and further studies with larger animal numbers may reveal that more differentially expressed metabolites from a pressure ulcer.

5. Limitations

Though our *Leporidae* model is intriguing and potentially of great use, there are several limitations to the present study that bear mention. First, the numbers of rabbits we used was small. The nature of this study was proof-of-concept, and we sought to answer a key questions (i.e., can we reproducibly measure skin metabolites from rabbits?) before proceeding to more detailed questions, which requires more animals. Our results are statistically significant, although we cannot rule out Type II statistical errors owing to a small sample size. Next, our sampling techniques allowed for detection of volatile and semi-volatile compounds produced by skin, but these compounds may not be significantly affected and engender only subtle metabolomic differences in skin affected by an early-stage pressure ulcer.

Also, the distributions of the detected compounds are invariably affected by the employed sorbent. We chose to use PDMS because it allows for capture of compounds with a broad range of polarities and ease of use, but its principle attraction was that it can be applied directly to the skin in a non-invasive fashion. However, it is possible that some compounds altered by pressure ulcer formation may not be efficiently captured and detected (especially if they cannot be volatilized). In particular, large, non-volatile compounds such as lipids would not be amenable for detection using the present methodology. It is also possible that greater changes could be induced for advanced pressure ulcers where the skin is broken, as opposed to early-stage ulcers.

Last, and fundamentally, the biology of pressure ulcers is not fully described in the research community and several tissue types are likely involved. As such, our analytic process may not be able to detect deeper tissue changes that could be key to pressure ulcer formation. However, the results are intriguing and perhaps with utilization of a broader range of analytical instrumentation (especially for non-volatile metabolites analysis) and identifying pressure ulcers at a slightly more advanced stage when skin surface breakdown begins, may be even more useful. In addition, pressure ulcers in humans are likely colonized by bacteria, as is most human skin. To minimize variability, we ensured minimal bacterial colonization by wiping the ear surface with 70% ethanol prior to metabolite sampling. This may have minimized the surface bacterial changes which may be a part of pressure ulcer pathophysiology. The bacterial contribution component of metabolome needs to be assessed independently in further studies. Despite these limitations, we believe that the *Leporidae* model is a promising tool in skin metabolome research and has a great potential to facilitate the understanding of chemical changes associated with various factors, including environmental exposures, or conditions, such as pressure ulcer formation.

6. Conclusion

The *Leporidae* model described here may serve as a good animal model of skin metabolome analysis. Its strengths lie in its convenience, cost-effectiveness, the robust amount of skin metabolites we identified, and its potential use to study disease states such as pressure ulcers. This model may facilitate identification of key metabolites from the outer layers of human skin that are present independent of multiple factors, including diet, lifestyle choices and environmental contaminants, though this remains to be tested. This may then inform human observational studies assessing how skin metabolites change over time and with disease states. In addition, a better understanding of skin metabolites (i.e., how to collect, identify, and validate them) may enhance an understanding of metabolites derived from non-skin sources such as breath, urine, or stool. Our ultimate vision is that skin metabolite analysis may eventually preclude skin biopsies and enable a complementary biologic assessment of human skin, and we believe that the model presented here may facilitate this process.

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Appendix

Table A1

Peak table generated from compounds identified from rabbit skin (both the ulcer and control rabbit ear groups).

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
2.0532	Ethanol	882	891	64-17-5	C ₂ H ₆ O	46	F U Br Bl Sa
2.2808	Butane	691	711	106-97-8	C ₄ H ₁₀	58	Br Bl
3.3015	Acetic acid	922	922	64-19-7	C ₂ H ₄ O ₂	60	F U Br Sk M Sa
3.6799	No ID						
3.9194	Ethane, 1,1-diethoxy-	930	930	105-57-7	C ₆ H ₁₄ O ₂	118	Sk
4.0352	No ID						
4.3259	Small carbonic acid						
4.3618	No ID						
4.4362	Likely a small carbonyl compound						
4.4771	Propanoic acid, 2,2-dimethyl-	868	868	75-98-9	C ₅ H ₁₀ O ₂	102	
4.5404	Hexanal	863	866	66-25-1	C ₆ H ₁₂ O	100	F U Br Sk M Bl Sa
4.8658	Possibly a carbonyl compound						
5.0828	No ID						

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
5.1383	Pentanoic acid	937	937	109-52-4	C ₅ H ₁₀ O ₂	102	F Br M Sa
5.2223	No ID						
5.3221	No ID						
5.4508	No ID						
5.4694	Ethanol, 2-butoxy-	706	751	111-76-2	C ₆ H ₁₄ O ₂	118	U Br
5.4778	No ID						
5.5910	No ID						
5.8310	Thujene	793	862	2867-05-2	C ₁₀ H ₁₆	136	F
5.9427	C5 aliphatic compound						
5.9771	C5 aliphatic compound						
6.0430	1-Pentanol, 3-methyl-	724	724	589-35-5	C ₆ H ₁₄ O	102	Br
6.0499	1-Heptanol, 4-methyl-	787	787	817-91-4	C ₈ H ₁₈ O	130	
6.1067	Branched hydrocarbon, C6						
6.1793	Phenylglyoxal	904	913	1074-12-0	C ₈ H ₆ O ₂	134	F
6.3994	Isooctanol	900	900	26952-21-6	C ₈ H ₁₈ O	130	
6.4490	1-Hexanol, 2-ethyl-	881	893	104-76-7	C ₈ H ₁₈ O	130	F U Br Sk M Sa
6.4843	Alkene or aldehyde, ~C8						
6.4889	Alkene or aldehyde						
6.5808	Octanal	835	835	124-13-0	C ₈ H ₁₆ O	128	F U Br Sk M Bl Sa
6.6633	No ID						
6.7241	No ID						
6.8049	3-ethyl-4-methylpentanol-1	880	892	100431-87-6	C ₈ H ₁₈ O	130	
6.8876	Branched alcohol, ~ C8						
6.9071	Unsaturated hydrocarbon, ~C8						
6.9590	Dihydropyran or Tetrahydropyridazine						
6.9792	1-Hexanol, 5-methyl-	804	813	627-98-5	C ₇ H ₁₆ O	116	

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
6.9861	Limonene	881	881	138-86-3	C ₁₀ H ₁₆	136	F U Br M Sa
7.0791	5-Methyl-1-heptanol	847	855	7212-53-5	C ₈ H ₁₈ O	130	
7.1484	No ID						
7.2254	No ID						
7.3856	No ID						
7.4458	Branched hydrocarbon, C9-C10						
7.5647	No ID						
7.7645	No ID						
7.7033	Possibly 3-ethyl-4-methylpentanol-1	782	808	100431-87-6	C ₈ H ₁₈ O	130	
7.7938	Isooctanol	831	848	26952-21-6	C ₈ H ₁₈ O	130	
7.8193	Aldehyde, ~C9						
7.9043	Unsaturated hydrocarbon						
7.9342	Unsaturated hydrocarbon						
7.9955	Benzoic acid, hydrazide or Benzoic acid, methyl ester						
8.0589	Nonanal	846	846	124-19-6	C ₉ H ₁₈ O	142	F U Br Sk M Sa
8.1072	Hydrocarbon						
8.2181	1-Heptanol, 2-propyl-	741	806	10042-59-8	C ₁₀ H ₂₂ O	158	
8.2761	No ID						
8.2913	Hydrocarbon						
8.4688	3,6,6-trimethyl-cyclohex-2-enone	842	842	78-59-1	C ₉ H ₁₄ O	138	U
8.5321	Pentanedioic acid, dimethyl ester	836	890	1119-40-0	C ₇ H ₁₂ O ₄	160	
8.7439	n-hydrocarbon						
8.8698	Normal long-chain alcohol						
8.9275	Benzoic acid	721	827	65-85-0	C ₇ H ₆ O ₂	122	F U Br Sk
9.0338	Alcohol, branched						
9.3037	Butanedioic acid, diethyl ester	858	909	123-25-1	C ₈ H ₁₄ O ₄	174	
9.5739	Cyclooctane, 1,4-dimethyl-, cis-	843	847	13151-99-0	C ₁₀ H ₂₀	140	
9.7325	1-Heptanol, 2-propyl-	872	888	10042-59-8	C ₁₀ H ₂₂ O	158	

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
9.9089	Decanal	822	861	112-31-2	C ₁₀ H ₂₀ O	156	F Br Sk M Sa
10.2849	No ID						
10.3028	No ID						
10.4709	No ID						
10.4188	Branched hydrocarbon						
10.6101	Z-Citral	823	823	106-26-3	C ₁₀ H ₁₆ O	152	
10.6855	Alcohol						
10.7971	Benzene, 1,3-bis(1,1-dimethylethyl)-	893	902	1014-60-4	C ₁₄ H ₂₂	190	F
10.8011	An isomer of Benzene, 1,3-bis(1,1-dimethylethyl)-, possibly Benzene, 1,4-bis(1,1-dimethylethyl)-						
10.8672	Branched alcohol						
11.0899	Alkene or alcohol						
11.1489	Benzaldehyde, 2,4-dichloro-	892	892	874-42-0	C ₇ H ₄ Cl ₂ O	174	
11.1725	Branched hydrocarbon						
11.2142	E-Citral	859	862	141-27-5	C ₁₀ H ₁₆ O	152	Sk Sa
11.2585	No ID						
11.3689	Pentanedioic acid, diethyl ester	880	949	818-38-2	C ₉ H ₁₆ O ₄	188	
11.4802	No ID						
11.5772	Oxalic acid ester with a long-chain alcohol						
11.5883	Long chain alcohol						
11.5936	Long chain hydrocarbon						
11.8192	2-tert-Butyl-5-methylbenzoquinone	720	762		C ₁₁ H ₁₄ O ₂	178	
11.8818	No ID						
12.0465	1,14-Tetradecanediol	810	810	19812-64-7	C ₁₄ H ₃₀ O ₂	230	
12.3277	No ID						
12.5532	Long chain normal aliphatic compound						
12.7090	Triacetin	857	864	102-76-1	C ₉ H ₁₄ O ₆	218	Sk
12.8241	Long chain hydrocarbon, e.g. Octadecane						
12.8765	Hexacosane	878	935	630-01-3	C ₂₆ H ₅₄	366	Sa
12.9823	1-Octanol, 2-butyl-	831	831	3913-02-8	C ₁₂ H ₂₆ O	186	Sa

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
13.1431	Myrcene	720	835	123-35-3	C ₁₀ H ₁₆	136	F Br Sa
13.2869	Phenol, 2-(1,1-dimethylethyl)-5-methyl-	748	780	88-60-8	C ₁₁ H ₁₆ O	164	
13.5778	Neryl acetate	722	746	141-12-8	C ₁₂ H ₂₀ O ₂	196	Sk
13.6927	2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo-	872	872	5888-33-5	C ₁₃ H ₂₀ O ₂	208	
13.8941	Tetradecene, E or Z isomers, uncertain double bond position					196	
14.0665	Long chain normal alkane						
14.0771	Long chain normal alkane						
14.3411	Long chain normal alkyne, C13 or greater						
14.3465	Long chain normal alkyne, C13 or greater						
14.5330	Hexadecane, 2,6,10,14-tetramethyl-	887	887	638-36-8	C ₂₀ H ₄₂	282	
14.7427	trans-Caryophyllene	780	823	87-44-5	C ₁₅ H ₂₄	240	F Sk M Sa
14.8069	1-Undecene, 4-methyl-	809	809	74630-39-0	C ₁₂ H ₂₄	168	Br
14.9564	Ethanone, 1,1'-(1,3-phenylene)bis-	807	814	6781-42-6	C ₁₀ H ₁₀ O ₂	162	
15.0726	Long chain aliphatic compound						
15.1732	Long chain normal aliphatic compound						
15.2985	1,2-Benzenedicarboxylic acid, dimethyl ester	830	851	131-11-3	C ₁₀ H ₁₀ O ₄	194	
15.5940	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	889	897	719-22-2	C ₁₄ H ₂₀ O ₂	220	
15.7430	No ID						
15.7980	Cyclic compound, C14 or greater						
15.9347	Aromatic compound						
16.0118	Long chain normal alcohol						
16.1158	Long chain normal aliphatic compound, likely contains oxygen						
16.2923	Long chain normal alkane						
16.5530	Phenol, 2,4-bis(1,1-dimethylethyl)-	897	899	96-76-4	C ₁₄ H ₂₂ O	206	
16.6613	Long chain alcohol or aldehyde, C12 or greater						

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
16.6709	Oxirane, tetradecyl-	818	820	7320-37-8	C ₁₆ H ₃₂ O	240	
16.9660	No ID						
17.2493	No ID						
17.3605	Dihexylsulfide	812	890		C ₁₂ H ₂₆ S	202	
17.5073	Branched alkane, C18 or greater						
18.8131	Long chain normal alcohol						
18.3669	Pentan-1,3-dioldiisobutyrate, 2,2,4-trimethyl-	798	807	6846-50-0	C ₁₆ H ₃₀ O ₄	286	
18.4601	1-Hexadecanol	785	806	36653-82-4	C ₁₆ H ₃₄ O	242	F Br Sk
18.4659	Alkene, C14 or greater						
18.6180	Long chain secondary alcohol						
18.8566	1(2H)-Naphthalenone, 3,4-dihydro-3,3,6,8-tetramethyl-	814	814	5409-55-2	C ₁₄ H ₁₈ O	202	
18.9628	1,14-Tetradecanediol	860	864	19812-64-7	C ₁₄ H ₃₀ O ₂	230	
19.4790	Methanone, diphenyl-	894	901	119-61-9	C ₁₃ H ₁₀ O	182	F Sk Sa
19.3656	Likely an ester or diester						
20.0195	No ID						
20.0726	No ID						
20.1673	No ID						
20.6899	No ID						
20.8155	Long chain normal alkane						
20.9373	Hexanoylthiocholine	802	812		C ₁₁ H ₂₄ NOS	218	
21.1803	1-Octadecyne	864	869	629-89-0	C ₁₈ H ₃₄	250	
21.3557	No ID						
22.0327	BHT-aldehyde	842	860	101100-38-3	C ₁₅ H ₂₂ O ₂	234	
22.2901	Benzyl Benzoate	794	794	120-51-4	C ₁₄ H ₁₂ O ₂	212	Sk
22.5664	Cycloalakanone or alcohol						
22.6787	Long chain normal alkane						
22.7651	Long chain normal alkane						
22.8923	No ID						
22.9927	Pentadecanal	867	879	2765-11-9	C ₁₅ H ₃₀ O	226	F
23.0585	No ID						
23.2378	No ID						
23.9643	Ethanone, 2,2-dimethoxy-1,2-diphenyl-	865	865	24650-42-8	C ₁₆ H ₁₆ O ₃	256	

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
24.0209	Hexanedioic acid, diethyl ester	730	735	141-28-6	C ₁₀ H ₁₈ O ₄	202	
24.1567	Long chain normal aliphatic compound, possibly alcohol						
24.2004	No ID						
24.2889	7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	788	830	82304-66-3	C ₁₇ H ₂₄ O ₃	276	F
24.4551	Long chain normal alkyne						
24.5101	Hexadecanoic acid, methyl ester	805	836	112-39-0	C ₁₇ H ₃₄ O ₂	270	Sk M Sa
25.3428	Long chain ester						
25.4179	Long chain normal alkane						
25.6875	Long chain normal aliphatic compound, possibly alkyne						
25.8510	Hexanedioic acid, bis(2-ethylhexyl) ester	744	770	103-23-1	C ₂₂ H ₄₂ O ₄	370	
26.5279	2-Hexyl-1-octanol	765	851	19780-79-1	C ₁₄ H ₃₀ O	214	
26.5424	9-Octadecenoic acid (Z)-, methyl ester	748	748	112-62-9	C ₁₉ H ₃₆ O ₂	296	
26.6444	NoID						
26.8175	Possibly long chain ester						
26.9845	No ID						
27.2958	Long chain normal carbonic acid						
27.5300	Long chain normal alkane, e.g. Eicosane						
27.6214	Long chain normal aliphatic compound, possibly alcohol						
27.6261	9-Octadecenoic acid	751	751	112-80-1	C ₁₈ H ₃₄ O ₂	282	
27.8091	Long chain normal aldehyde						
27.8703	No ID						
27.9945	No ID						
28.3033	Possibly an ester						
28.4259	1-Hexadecanol, 2-methyl-	756	756	2490-48-4	C ₁₇ H ₃₆ O	256	
28.7777	Possibly a diester						
28.8544	Large alkane						
28.9541	No ID						
29.0925	Hexanedioic acid, bis(2-ethylhexyl) ester	756	820	103-23-1	C ₂₂ H ₄₂ O ₄	370	
29.1208	Hexanedioic acid, mono(2-ethylhexyl)ester	796	811	4337-65-9	C ₁₄ H ₂₆ O ₄	258	

RT (min)	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW	Human VOC
29.1528	Large branched alkane						
29.7807	Long chain alcohol						
30.1915	No ID						
30.4667	11-Octadecenal	727	727	56554-95-1	C ₁₈ H ₃₄ O	266	

Compounds were selected after all metabolite data was compiled, deconvoluted, and background metabolites (i.e., room air and rabbit cage blank controls) were excluded. RT – retention time; Match – NIST MS Search forward match factor for the compound; Reverse Match – NIST MS Search reverse match factor for the compound (the match factor obtained by ignoring all peaks that are in the sample spectrum but not the library spectrum); CAS – Chemical Abstracts Service identification number; MW – molecular weight; F – feces; U – urine; Br – breath; Bl – blood; Sk – skin; M – breastmilk; Sa – saliva

Figure 1. PDMS patches placed over the ventral ear surface. These were then covered with PTFE and Tegaderm® (not shown).

Figure 2.
Magnet set up on rabbit ear. Note the position between the central artery (center of ear running longitudinal) and marginal vein (outer edge).

Figure 3.
Early-stage ulcer immediately after magnet removal. The white circle turned into a non-blanching erythematous lesion within 60 sec (not shown).

Figure 4.

A representative example chromatogram illustrating the compounds detected from the PDMS patches placed on intact rabbit skin. A portion of the chromatogram is enhanced (inset) to illustrate the richness of chemical composition of the skin sample. The high abundance peaks are due to siloxanes bleeding from septa and PDMS patches.

Table 1

Rabbit skin metabolites categorized according to the functional group. Compounds were selected after all metabolite data was compiled, deconvoluted, and background metabolites (i.e., room air and rabbit cage blank controls) were excluded.

Compound Class	Identified detected compounds
Hydrocarbons, saturated and unsaturated, including terpenes, cyclic and branched	Butane Thujene Limonene Cyclooctane, 1,4-dimethyl-, cis- Hexacosane Myrcene Tetradecene Hexadecane, 2,6,10,14-tetramethyl- Caryophyllene 1-Undecene, 4-methyl- 2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)- 1-Octadecyne
Aliphatic alcohols, normal and branched	Ethanol 1-Pentanol, 3-methyl- 3-ethyl-4-methylpentanol-1 1-Heptanol, 4-methyl- 1-Hexanol, 5-methyl- 5-Methyl-1-heptanol Isooctanol 1-Hexanol, 2-ethyl- 1-Heptanol, 2-propyl- 1,14-Tetradecanediol 1-Octanol, 2-butyl- 1-Hexadecanol 1,14-Tetradecanediol 2-Hexyl-1-octanol 1-Hexadecanol, 2-methyl-
Aliphatic aldehydes, normal	Hexanal Octanal Nonanal Decanal E, Z-Citral Pentadecanal 11-Octadecenal
Ketones, aliphatic and aromatic	3,6,6-trimethyl-cyclohex-2-enone 2-tert-Butyl-5-methylbenzoquinone 1(2H)-Naphthalenone, 3,4-dihydro-3,3,6,8-tetramethyl- Methanone, diphenyl-
Ethers, mono and di-	Ethane, 1,1-diethoxy- Pentanedioic acid, dimethyl ester Butanedioic acid, diethyl ester 9-Octadecenoic acid (Z)-, methyl ester
Carbonic acids, aliphatic	Acetic acid Pentanoic acid Propanoic acid, 2,2-dimethyl- Benzoic acid 9-Octadecenoic acid
Esters, aliphatic and aromatic	Benzoic acid, methyl ester Pentanedioic acid, diethyl ester Triacetin Neryl acetate 2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo- 1,2-Benzenedicarboxylic acid, dimethyl ester Hexanedioic acid, diethyl ester Hexadecanoic acid, methyl ester Hexanedioic acid, bis(2-ethylhexyl) ester Benzyl Benzoate

Compound Class	Identified detected compounds
	Hexanedioic acid, mono(2-ethylhexyl)ester
Aryls	Benzene, 1,3-bis(1,1-dimethylethyl)- Benzene, 1,4-bis(1,1-dimethylethyl)-
Heterocyclic compounds	Dihydropyran Oxirane, tetradecyl- 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione
Sulfur-containing compounds	Dihexylsulfide
Multiple functional groups (aliphatic and aromatic compounds), including heteroatoms	Ethanol, 2-butoxy- Phenylglyoxal Benzaldehyde, 2,4-dichloro- Ethanone, 1,1'-(1,3-phenylene)bis- Phenol, 2-(1,1-dimethylethyl)-5-methyl- Phenol, 2,4-bis(1,1-dimethylethyl)- Pentan-1,3-dioldiisobutyrate, 2,2,4-trimethyl- Hexanoylthiocholine 3,5-di-tert-butyl-4-hydroxybenzaldehyde Ethanone, 2,2-dimethoxy-1,2-diphenyl-

Table 2

List of 12 compounds from rabbit skin identified to be grossly different between ulcer and control skin.

Compound Number	RT (Table 1)	t-test adjusted p-value	FC	Chemical Name/Tentative Chemical Identification	Match	Reverse Match	CAS	Formula	MW
1	4.4771	0.4545	0.9	Propanoic acid, 2,2-dimethyl-	868	868	75-98-9	C5H10O2	102
2	6.8049	0.1736	0.7	3-ethyl-4-methylpentanol-1	809	823	100431-87-6	C8H18O	130
3	6.8876	0.0005	0.2	Branched alcohol, ~ C8					
4	6.9071	0.2265	0.8	Unsaturated hydrocarbon, ~ C8					
5	6.9792	0.5590	0.7	1-Hexanol, 5-methyl-	804	813	627-98-5	C7H16O	116
6	8.4688	0.1682	0.7	3,6,6-trimethyl-cyclohex-2-enone	842	842	78-59-1	C9H14O	138
7	8.7439	0.4534	0.8	n-hydrocarbon					
8	10.7971	0.1736	0.6	Benzene, 1,3-bis(1,1-dimethylethyl)-	893	902	1014-60-4	C14H22	190
9	11.8818	0.1682	0.7	No ID					
10	15.5940	0.1736	0.7	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	889	897	719-22-2	C14H20O2	220
11	17.5073	0.9307	0.9	Branched alkane, C18 or greater					
12	18.4659	0.8368	1	Alkene, C14 or greater					

When these compounds were analyzed for statistical significance, one compound, tentatively identified as a C8 branched alcohol (**bold**), was present in significantly higher amount (5-fold difference) in the ulcer group compared to the intact skin group. RT, retention time; FC, fold-change from the intact rabbit ears to the ulcerated ears; MW, molecular weight