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Research article

Molecular analysis of human tick-bitten skin yields signatures associated with distinct spatial and temporal trajectories - A proof-of-concept study

Wenna Lee [a,b,c,**], Rym Ben-Othman [b], Patrycja Skut [b], Amy Huey-Yi Lee [d], Amanda D. Barbosa [a,f,g], Miles Beaman [e], Andrew Currie [c,h], Nathan T. Harvey [e,i], Prasad Kumarasinghe [j,k,l], Roy A. Hall [m], James Potter [m], Stephen Graves [n], Nicholas P. West [o], Amanda J. Cox [o], Peter J. Irwin [f], Tobias R. Kollmann [b,1,***], Charlotte L. Oskam [a,c,*,1]

[a] *Centre for Biosecurity and One Health, Harry Butler Institute, Murdoch University, Murdoch, WA, Australia*
[b] *Telethon Kids Institute, Perth, WA, Australia*
[c] *School of Medical, Molecular, and Forensic Sciences, College of Environmental and Life Sciences, Murdoch University, Murdoch, WA, Australia*
[d] *Molecular Biology and Biochemistry, Simon Fraser University, British Columbia, Canada*
[e] *Faculty of Health and Medical Sciences, Pathology & Laboratory Medicine, University of Western Australia, Perth, WA, Australia*
[f] *School of Veterinary Medicine, College of Environmental and Life Sciences, Murdoch University, Murdoch, WA, Australia*
[g] *CAPES Foundation, Ministry of Education of Brazil, Brasilia, DF, Brazil*
[h] *Centre for Molecular Medicine and Innovative Therapeutics, Health Futures Institute, Murdoch University, Murdoch, WA, Australia*
[i] *Department of Anatomical Pathology, PathWest Laboratory Medicine, QEII Medical Centre, Perth, WA, Australia*
[j] *School of Medicine, University of Western Australia, Crawley, WA, Australia*
[k] *College of Science, Health, Education and Engineering, Murdoch University, Murdoch, WA, Australia*
[l] *Western Dermatology, Hollywood Medical Centre, Nedlands, WA, Australia*
[m] *Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD, Australia*
[n] *Australian Rickettsial Reference Laboratory, Barwon Health, Geelong, VIC, Australia*
[o] *School of Pharmacy and Medical Sciences, and Menzies Health Institute, Griffith University, QLD, Australia*

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ABSTRACT

Tick-associated diseases present challenges due to tridirectional interactions among host-specific responses, tick toxins and salivary proteins as well as microbes. We aimed to uncover molecular mechanisms in tick-bitten skin samples (cases) and contralateral skin samples (controls) collected simultaneously from the same participants, using spatial transcriptomics. Cases and controls analysed using NanoString GeoMx Digital Spatial Profiler identified 274 upregulated and 840 downregulated differentially expressed genes (DEGs), revealing perturbations in keratinization and immune system regulation. Samples of skin biopsies taken within 72 h post tick-bite DEGs had changes in protein metabolism and viral infection pathways as compared to samples taken 3

* Corresponding author. Centre for Biosecurity and One Health, Harry Butler Institute, Murdoch University, Murdoch, WA, Australia.
** Corresponding author. Telethon Kids Institute, Perth, WA, Australia.
*** Corresponding author. Telethon Kids Institute, Perth, WA, Australia.
E-mail addresses: Wenna.Lee@telethonkids.org.au (W. Lee), Tobias.Kollmann@telethonkids.org.au (T.R. Kollmann), C.Oskam@murdoch.edu.au (C.L. Oskam).
[1] Co-senior author.

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Table 1
Tick-bitten participants details.

Patient ID	Main Cohort					Sub-cohort 1 **No control biopsy**	Sub-cohort 2 long-term response
	AHE	**AHL**	**AHN**	**AHR**	**AJS**	**AHP**	**AJK**
Sex	M	M	F	M	M	F	M
Age	22	58	56	67	65	53	68
Local symptoms	No	No	1. Redness around the bite	1. Redness around the bite 2. Itchiness	1. Redness around the bite 2. Itchiness	1. Redness around the bite 2. Itchiness	1. Redness around the bite 2. Itchiness 3. Occasional discharge at tick bite site (clear or cloudy fluid, slightly blood stained at times)
Generalised symptoms	No	No	No	"I appear to develop gut discomfort following a tick bite."	No	No	No
Past tick bites	5+	4	5	4	5+	5+	5+
Tick attachment location	Abdomen	Back/shoulders	Back/shoulders	Arm/armpit	Legs	Head/face	Arm/armpit
Tick attachment time	6–24 h	>24 h	>24 h	>24 h	>24 h	>24 h	6–24 h (3 months previously)
Tick species	*Amblyomma triguttatum*	*Amblyomma triguttatum*	*Amblyomma triguttatum*	*Amblyomma triguttatum*	*Amblyomma triguttatum*	*Amblyomma triguttatum*	Not available
Tick instar	Nymph	Adult female	Nymph	Adult male	Larvae	Nymph	Not available
CRP (mg/L)	2	8	36	<1	4	5	1
Haematology & Blood Chemistries interpretation	no significant pathology	no significant pathology	no significant pathology	no significant pathology	pathological liver changes associated with tick bite.	no significant pathology	no significant pathology
Serology to bacteria: interpretation	no significant serology	pre-existing antibodies to Spotted Fever Group *Rickettsia*. Seroconversion to *Coxiella burnetii* at 3 months post tick-bite	no significant serology	no significant serology	no significant serology	no significant serology	no significant serology
Serology to known tick virus	Negative	Negative	Negative	Weak positive at 1-week post-tick bite	Negative	Negative	Negative
MAVRIC on tick patient culture	Negative	Weak positive	Negative	Negative	Negative	Negative	Negative
Bacterial TOI in tick-bite skin	Nil	Rickettsiaceae	*Rickettsia*	Nil	*Rickettsia*	N/A	Nil
Bacterial TOI in control skin	Nil	Nil	Nil	Nil	Nil	N/A	Nil
Bacterial TOI in biting tick	*Rickettsia* *Francisella*	*Rickettsia* *Francisella*	*Rickettsia* *Francisella*	*Rickettsia* *Francisella*	*Rickettsia* *Francisella*	*Rickettsia* *Francisella*	N/A

[a]Taxa of interest, TOI; [b]monoclonal antibodies to viral RNA intermediates in cells, MAVRIC; [c]not applicable, N/A; [d]C reactive protein, CRP; [e]double-stranded, ds; antibodies not detected, Negative; no DNA of TOI detected, Nil.

months post tick-bite, which instead displayed significant perturbations in several epigenetic regulatory pathways, highlighting the temporal nature of the host response following tick bites. Within-individual signatures distinguished tick-bitten samples from controls and identified between-individual signatures, offering promise for future biomarker discovery to guide prognosis and therapy.

1. Introduction

Climate change and human usage of natural habitats are expected to increase tick-borne diseases (TBDs), which the Centers for Disease Control and Prevention of the United States of America have already described a two-fold spike within a span of 13 years, representing 77 % of all reported vector-borne diseases (VBDs) [1,2]. Throughout the world, tick-borne microbial pathogens (TBPs) such as bacteria, viruses and protozoa, have been rigorously investigated using the guidelines of Koch's postulates and have confirmed the causative agents of many TBDs [3–5]. In the last 50 years, considerable success has been achieved in identifying TBPs [6], understanding tick envenomation [7], and in the understanding of specific hypersensitivity reactions [8]. Significant gaps in knowledge remain, however, and there are still tick-associated pathologies that have yet to be determined because current methodologies rely predominantly on the detection of TBPs in patients presenting tick-associated symptoms [9,10]. Ill-defined tick-associated illness, such as post-treatment Lyme disease syndrome (PTLDs) [11] and Debilitating Symptom Complexes Attributed to Ticks (DSCATT) [12], comprise heterogenous symptoms that can persist long after tick bite despite no detection of a pathogen. In Australia, there is an increasing urgency to bridge the knowledge gaps because there are many individuals suffering from tick-associated illnesses resembling Lyme disease symptomology but no evidence of *Borrelia burgdorferi*, the causative pathogen) [12].

Host-tick and/or host-microbe interactions in the tick-bitten skin represent the initiating epicentre of the bite and can affect the trajectory of pathogenesis [13–18]. Specifically, the skin is an important site of the complex and dynamic interactions between host-specific defences and territorial invasion by the biting arthropod and introduced pathogens [13,14,17]. For example, the host adaptive immune response can be activated to elicit tick immunity, causing the arthropod to disengage prematurely (before feeding to repletion) and by mobilising immune cells into the bite site to phagocytose inoculated pathogens [16,19,20]. However, the host immune response has also been shown to be modulated by tick saliva as part of an "arms race" to prevent disruption to the tick's blood meal, consequently enabling inoculated TBPs to replicate in resident skin cells, such as keratinocytes and epidermal dendritic cells [13,14,17]. Indeed, the ability of ticks to dampen host immune responses and to evade immune detection, allowing the host to remain asymptomatic, has rendered the tick-bitten skin a biological reservoir for TBPs such as *B. burgdorferi* and tick-borne encephalitis virus [18,21]. The species-specific and host-specific nature of tick-mediated immune modulation and immune responses, respectively, further complicate the nature of the interaction at the tick bite site, resulting in heterogeneity in pathology and symptomology [13]. Investigating host perturbations at the tick-bitten skin, which has thus far been inadequately studied, may add fresh knowledge of these processes and likely uncover molecular targets that have the potential to be exploited to benefit the human.

For this technical pilot study, tick bitten participants were enrolled in Western Australia during the 2021/2022 tick season (spring and summer) and consented to provide skin samples for spatially resolved, untargeted transcriptomic analysis. Paired biopsies (at tick bite site and the unaffected corresponding contralateral site) presented a unique opportunity to investigate the pathophysiology of tick bites in the skin in comparison to healthy skin obtained from the same individual. Apart from elucidating between-individual heterogeneity in immune responses, tick bite differentially expressed genes (DEGs) and enriched pathways, our approach also allowed detection of spatially and temporally resolving host specific disturbances. The findings of this technical pilot study confirmed that the methodology could be applied in future studies with larger sample size to pinpoint pathogenic mechanisms as well as identify possible targets for diagnosis, prognosis, or therapeutic interventions.

2. Results

2.1. Participant cohort and power considerations

In total, seven participants were enrolled. Four males and one female bitten by a tick in the preceding 72 h ('acute' bite) were enrolled for the technological pilot cohort and volunteered paired biopsies and blood samples. An additional female participant (AHP), enrolled under the same conditions as the main cohort, declined a control biopsy after the biopsy at the tick bite site was collected and was enrolled into sub-cohort 1. Furthermore, participant (AJK) who was bitten three months prior (long-term response) was enrolled into sub-cohort 2. The meta-data of all seven participants are summarised in Table 1. All biting ticks were identified as *Amblyomma triguttatum.* With respect to the taxa of interest (TOI), bacterial 16S rRNA sequencing of the ticks revealed DNA from the genus *Rickettsia* and *Francisella*, as expected, in addition to other genera comprising the tick's microbiome. Only three tick-bitten skin biopsies had detectable DNA from the family Rickettsiaceae, and among them, two could be taxonomically classified within the genus *Rickettsia* (Table 1). No other TOI were detected in the skin biopsies.

Furthermore, we note that with our approach, where each participant is contrasted to themselves, statistically significant changes should be detectable in fewer than five participants [22,23]. Thus, while we consider this pilot of spatial genomics applied to tick bites strictly exploratory, it is likely to provide sufficiently robust insight to assess the feasibility of this approach for future studies.

2.2. Cellular infiltrates in tick bitten skin

Visual comparison of H&E stains between paired sections (tick-bitten cases and contralateral controls) showed that tick-bitten sections had significant cellular infiltrates and increased cellular density when compared to the healthy controls (Figs. 2 and 3). In the haematoxylin-rich staining in the dermal area close to the epidermis, depicted in Fig. 3d, the cellular infiltrate appeared to be dominated by CD45 leukocytes and CD3 marker which successfully identified T cells (Fig. 3f).

2.3. Spatial transcriptional patterns

As shown in Fig. 3 in a representative tick bitten sample the observable increased cellular density in comparison to control sections corresponded to increased transcriptional activity (Fig. 4b). Spatial deconvolution analysis was conducted to bioinformatically explore the cellular composition of each region of interest (ROI) using gene expression data. The ROIs obtained from the control skin samples clustered together on a heatmap of a cell-specific transcriptional density with fewer counts per surface area, while tick-bitten ROIs had more upregulated transcription and clustered together towards the right side of the heatmap (Fig. 4a). Greater expression of gene transcripts from naïve CD8 T cells, neutrophils, natural killer (NK) cells and plasma cells were observed in tick-bitten ROIs than compared with healthy controls (Fig. 4a). Furthermore, while tick-bitten deep and dermal ROIs tended to cluster together with between-patient variability, epidermal ROIs clustered by participant, regardless of state (tick-bitten cases and contralateral controls) (Fig. 4a). Within tick-bitten ROIs, study participant AJK, who was bitten 3 months prior to recruitment (Table 1), demonstrated higher transcript levels expressed by naïve B cells, CD4 memory T cells and Treg cells from ROIs obtained from the dermal region when compared to dermal ROIs obtained from acutely bitten participants (Fig. 4a).

In Fig. 4b, where the ROIs were arranged by decreasing cellular density from the left, the 12 most cellularly dense ROIs comprised tick-bitten samples (left of the red vertical line), with half comprising dermal ROIs from chronic participant AJK (purple arrows). Thereafter the cellular density gradually tapered off to the right of the red line. The range of cellular density was greater in tick-bitten ROIs (black violin), reaching above 0.026 cells/μm^2 while a smaller range of cellular density of up to 0.010 cells/μm^2 was observed in healthy control ROIs (green violin) (Fig. 4b). A Wilcoxon test confirmed these differences as statistically significant with a p-value of 1.25e-04. In Fig. 4c, tick-bitten ROIs displayed a more consistent distribution of immune cells and a more substantial proportion of Treg cells (pink) than control ROIs. The cellular composition of control ROIs (Supplemental Table 2) was more varied (Fig. 4c) and demonstrated a higher proportion of fibroblasts (grey) and endothelial (brown) cells (Fig. 4c). From the heatmap (Fig. 4a), the control ROIs demonstrated an overall lower transcript count per area expressed by fibroblasts and endothelial cells, however, due to the lower cellular density (Fig. 4b), fibroblasts and endothelial cells contributed a higher proportion (Fig. 4c). Study participants AHL, AJS and AHN who all had detectable Rickettsiaceae and *Rickettsia* via bacterial 16S rRNA amplicon sequencing (Supplemental Table 1) contained up to 30 % of mast cells (yellow) regardless of whether the sections were from tick bitten or control skin sites (Fig. 4c). Power calculations in spatial transcriptomics are a relatively new area in research and require further investigation because, unlike bulk RNA

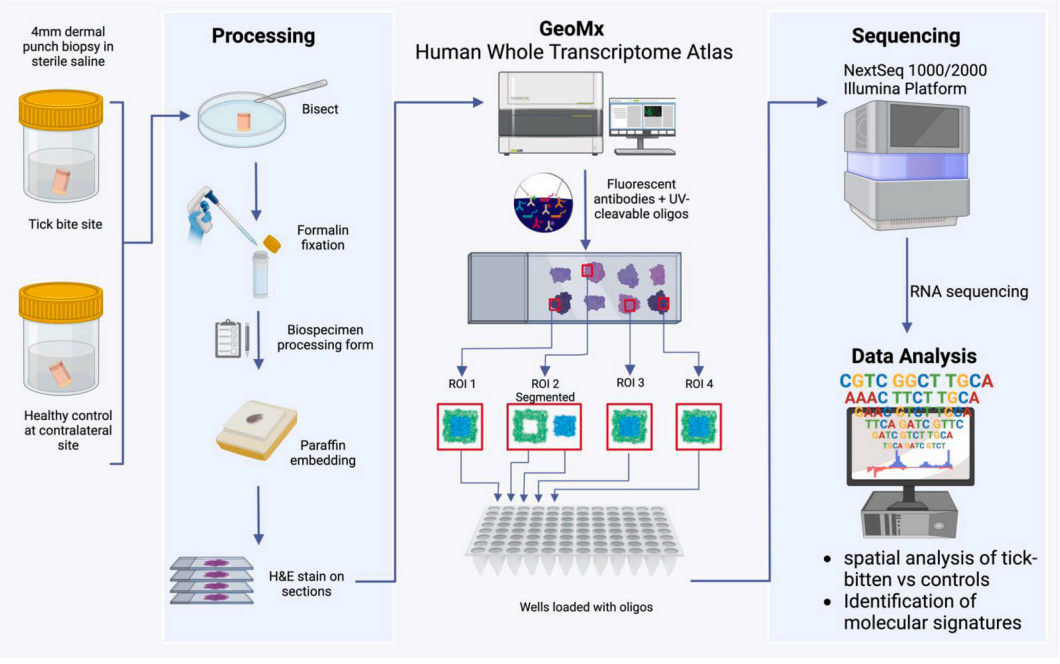

Fig. 1. Spatial transcriptomics workflow used in this study: Skin tissue collection, processing, and histology to GeoMx spatial transcriptomics, sequencing, and data analysis. Created with BioRender.com.

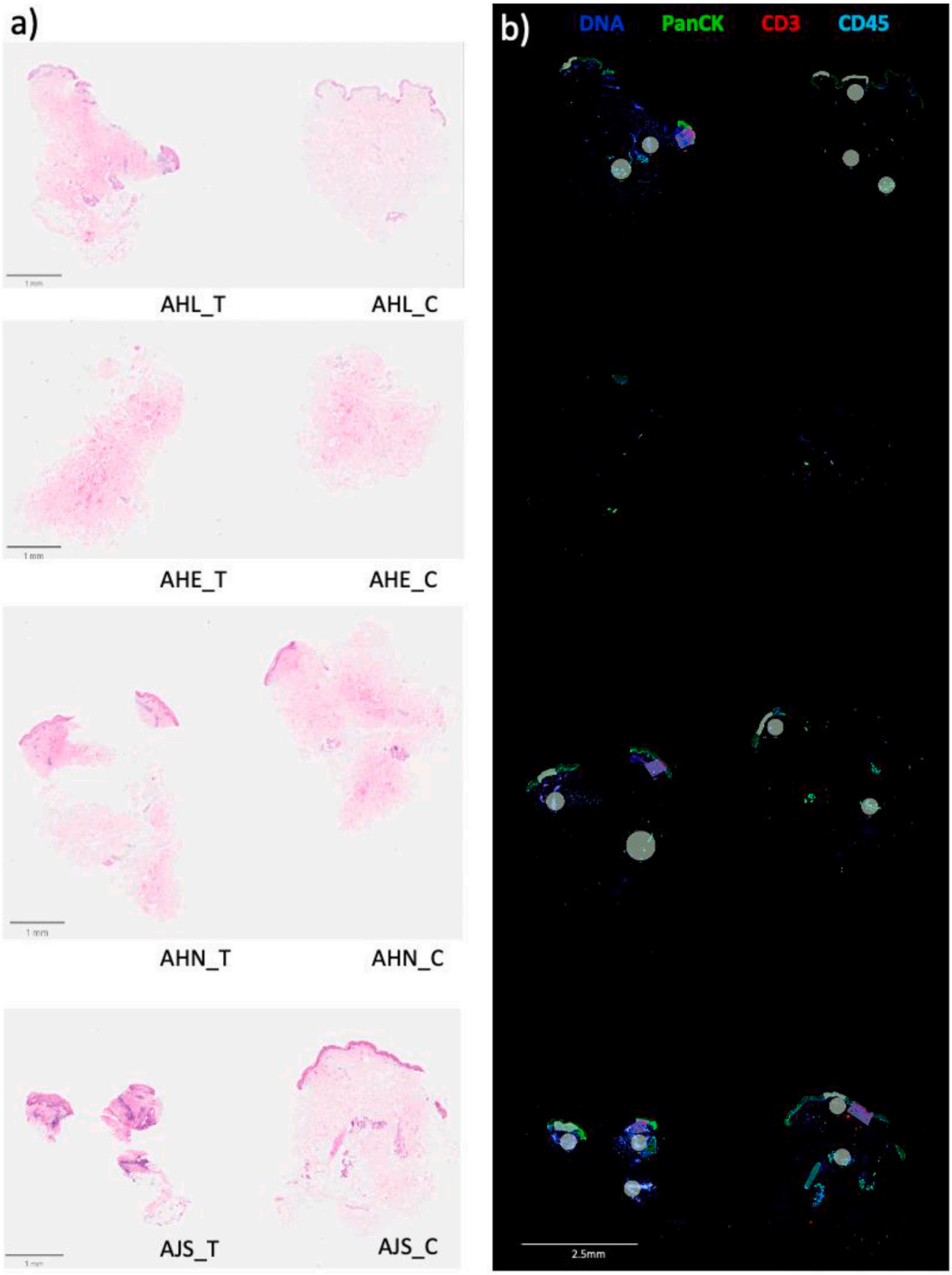

Fig. 2. Cellular infiltrates in tick bitten skin with representative sections of tick bitten participants, top down, left to right: AHL Tick bitten; AHL Control; AHE Tick bitten; AHE Control; AHN Tick bitten; AHN Control; AJS Tick bitten; AJS Control. a) Haematoxylin & eosin stain for histological annotation; b) Region of Interest (ROI) selection slide with morphology markers: SYTO13 DNA (Blue), PanCK (Green), CD3 (Red) and CD45 (Cyan). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sequencing, each tissue contains pseudo-duplicates and variable ROI sizes [23]. As a feasibility study without a clinical endpoint, no formal sample size analysis was undertaken, and despite being a technical pilot, the sample size aligns with other published research [22,23].

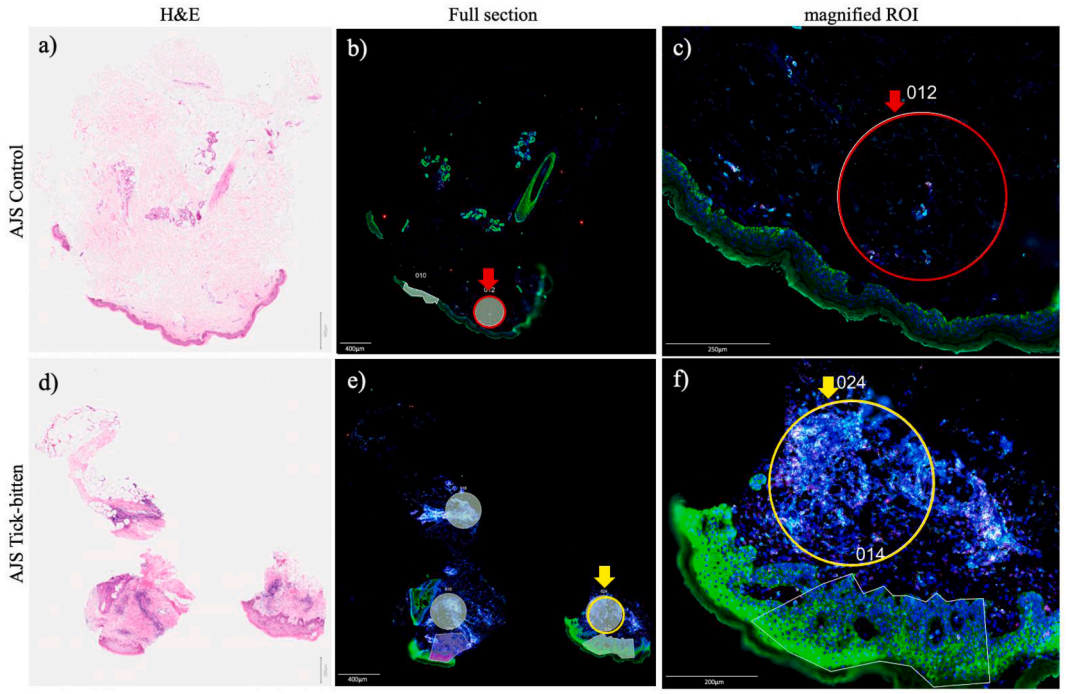

Fig. 3. Cellular infiltrates in tick bitten skin with section comparison for participant AJS, top row control biopsy and bottom row tick-bitten biopsy. From left to right: H&E stain, full section, and magnified ROI selection. a) AJS full control section H&E; b) AJS full control section morphologically marked, selected dermal ROI (red arrow); c) AJS control section ROI selection magnified (red arrow); d) AJS tick bitten full section H&E; e) AJS tick bitten full section morphologically marked, selected dermal ROI (yellow arrow); f) AJK tick-bitten section ROI selection magnified (yellow arrow). For b, c, e, f morphology markers used SYTO13 DNA (Blue), PanCK (Green), CD3 (Red) and CD45 (Cyan). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4. Distinct gene expression profile in tick-bitten skin when compared to controls

There were 1,114 DEGs identified when the transcripts in all tick-bitten skin sections were compared to the transcripts obtained from contralateral control skin sections, regardless of location in the skin. From the 274 upregulated and 840 downregulated DEGs, 10 enriched pathways were identified, namely 'Keratinization' and 'Formation of the cornified envelope', and 'Neutrophil degranulation' and 'Interleukin-4 and Interleukin-13 signalling' (Fig. 5). All DEGs, their fold change and the adjusted p-values are listed in Supplemental Table 2. The keratinization pathway was identified as the most significantly modulated pathway, with an adjusted p-value of 8.72e-09 and comprising 43 DEGs (19 up and 24 down), while the neutrophil degranulation pathways consisted of the highest number of DEGs with 22 up and 39 down and an adjusted p-value of 4.58e-05 (Fig. 5a and b). All significant pathways, adjusted p-values and DEGs are listed in Supplemental Table 3. The functional enrichment visualisation plot shows that the upregulated genes in the keratinization pathway were not only involved in keratinocyte cellular differentiation but also in upregulation of genes associated with antimicrobial peptides and cell-to-cell adhesion molecules associated with neutrophilic degranulation (Fig. 5c and d). Thus, the two top-most impacted pathways (keratinization and neutrophil degranulation) functionally interact.

2.5. The dermis identified as the most transcriptionally active site during tick bites

When the different locations were analysed separately, the dermis, with 1,869 DEGs, appeared as the most transcriptionally active location, followed by the deep dermis with 722 DEGs, and the epidermis with only a single DEG (Fig. 6a). The majority of DEGs were identified in ROIs from the dermal layer. Therefore, we concentrated our analysis on the dermal layer. From the 497 upregulated and 1,372 downregulated DEGs identified in the dermis, 18 enriched pathways were identified that were part of haemostasis and olfactory signalling processes (Fig. 6). Haemostasis related pathways 'Platelet degranulation' and 'Response to elevated platelet cytosolic Ca2+', were identified as the most significant, both with 37 DEGs and adjusted p-values of 1.42e-05 and 2.16e-05 respectively (Fig. 6b and c). The two pathways with the highest number of DEGs, 'Expression and translocation of olfactory receptors' and 'Olfactory Signalling Pathway', were part of the olfactory signalling process, both with gene counts of 74. From the functional enrichment plot (Fig. 6c), the haemostasis pathways were functionally associated and shared common DEGs with the extracellular matrix organisation and signalling pathways while the two olfactory signalling pathways formed a functionally discrete cluster. From the 150 upregulated and 572 downregulated DEGs identified in the deep dermis, the same two olfactory signalling process pathways as in the dermis were found, each with a gene count of 32 and an adjusted p-value of 1.03e-02.

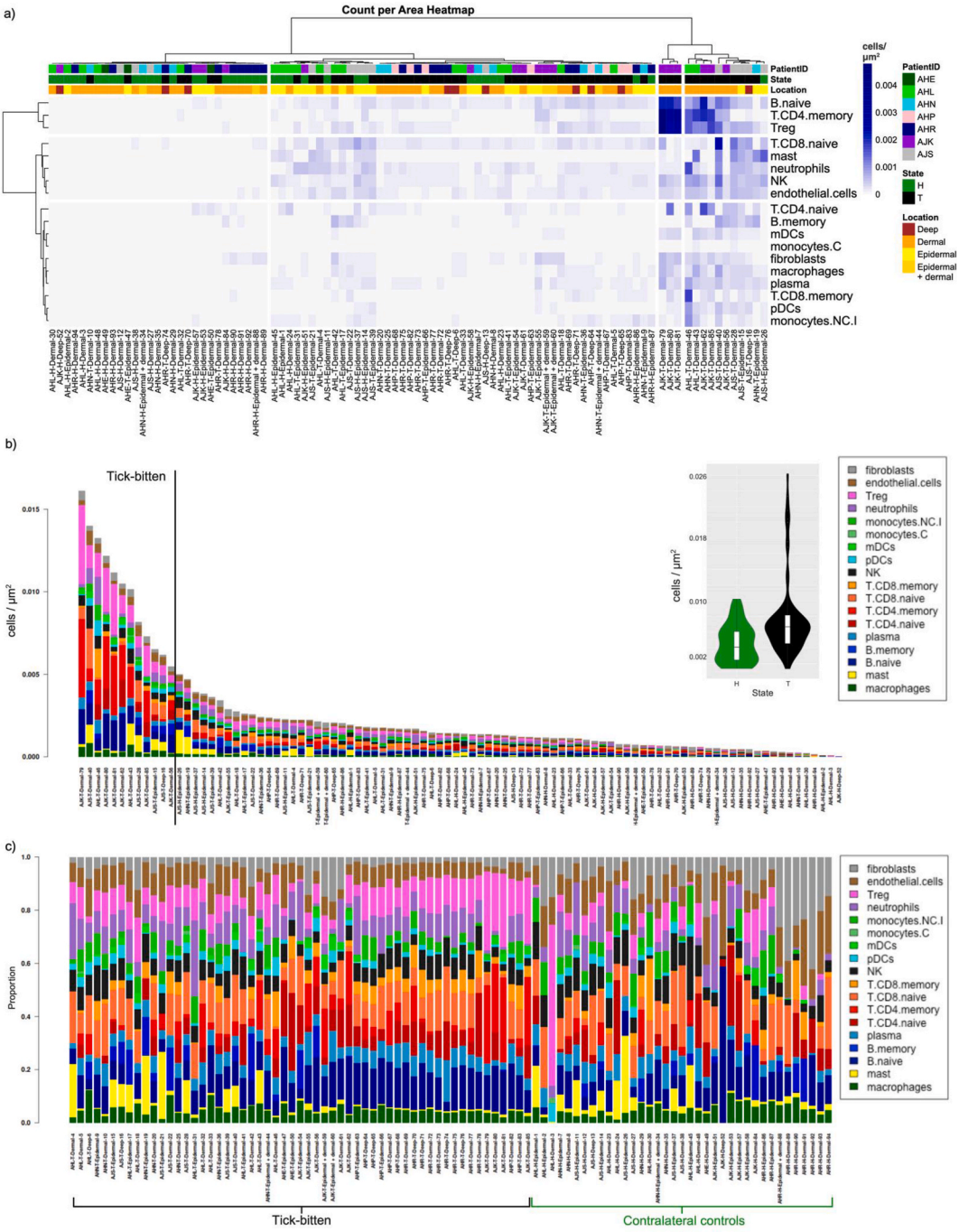

Fig. 4. Spatial transcriptional patterns of all ROIs based on gene expression data using Ward D clustering with a Euclidean distance between columns and rows. a) Heatmap of the spatial deconvolution of ROIs based on cell-specific transcript counts per area; b) Hierarchy of ROI cell density (cells/ μ m^2) with 12 most cellularly dense ROIs comprised tick-bitten samples (left of the black vertical line) and corresponding violin plot comparing total cell density from healthy (H) control (green violin) and tick-bitten (T) ROIs (black violin); c) Cellular composition of ROIs as a proportion of total cell density. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. Acute vs non-acute participants display strikingly different gene expression patterns

All paired biopsies analysed, one chronic and five acutely-bitten participants, showed DEGs with a *P*-value of 0.05 and fold change of 1.5 in either direction (Fig. 7a) (Full list of DEGs available in Supplemental Table 2. However, when a false discovery rate correction

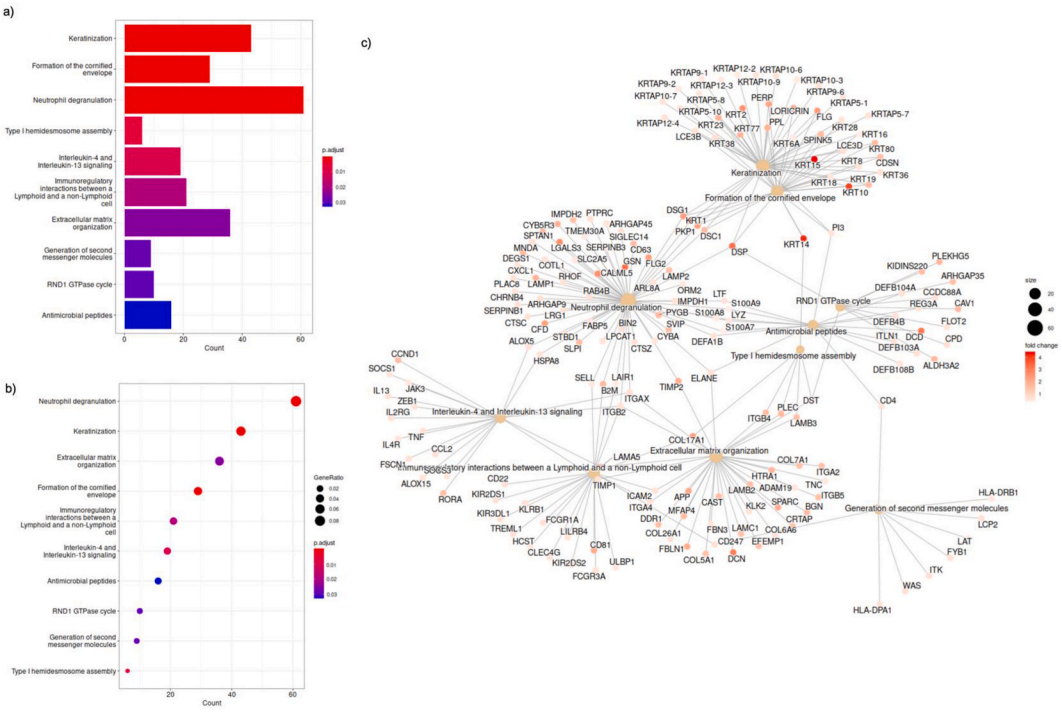

Fig. 5. Distinct gene expression profile in tick-bitten skin when compared to controls. Differentially expressed gene analysis from tick-bitten skin. a) Bar plot of the significant pathways in tick-bitten skin ranked by significance; b) Dot plot of the significant pathways in tick-bitten skin ranked by gene count; c) Functional enrichment visualisation of pathways found in tick bitten skin.

of 0.05 was applied to account for the multivariate analysis, only participants AHL, AHN, AHR and AJK had DEGs between the tick bite and control sites that achieved significance (Fig. 7a). AHL had 15 DEGs identified (one upregulated and 14 downregulated), AHN had 92 DEGs identified (47 upregulated and 45 downregulated), AHR had 4,716 DEGs identified (1389 upregulated and 3,327 downregulated), and AJK had 1,014 DEGs identified (440 upregulated and 574 downregulated). The DEGs identified in each participant were compared to ascertain similarities and used to identify enriched pathways (Fig. 7b).

Acutely-bitten participants AHR and AHN shared 36 common pathways, 14 of which were for the metabolism of proteins, such as eukaryotic translation elongation and termination, peptide chain elongation, and viral infection pathways such as viral mRNA translation (Fig. 7b and c). Participant AJK, bitten 3 months prior, had 80 pathways identified that were associated with epigenetic regulation of gene expression pathways, such as DNA methylation and PRC2 methylates histones and DNA (Fig. 7b and c). This participant's symptomology differed in some respects from the acutely bitten participants (Table 1) and included an occasional yet persistent, fluid discharge at the site of the original tick bite.

2.7. Feasibility

This technical pilot confirmed that spatial transcriptomics is an informative tool to detect molecular signatures associated with temporal trajectories in human skin. Despite challenges with participant recruitment (including those due to COVID lockdown measures), enrolled participants were highly willing to volunteer blood and skin biopsy samples despite the relatively invasive dermal punch biopsy procedure. Our pilot also showed placing multiple sections (Fig. 2) (up to 8 sections of 4 mm biopsies) on a single microscope slide and selecting ROIs from informative locations into the final (more expensive) analysis was feasible and can significantly lower costs.

3. Discussion

Tick bite-associated diseases display heterogeneous symptomology; insights into the underlying mechanisms are constrained by gaps in knowledge [9,10]. To fill this gap, we conducted a technological pilot study of spatial transcriptomics that was able to successfully generate transcriptomic data from distinct anatomic regions of biopsy samples and clearly demonstrated unique transcriptomic profiles both between regions in the tissue as well as in the tick-bite samples. These outcomes support that spatial transcriptomic approaches have the potential to deliver relevant insights and possibly support identification of diagnostic and prognostic biomarkers, as well as identify potential targets for therapeutic interventions. Specifically, we found differences in transcriptional activity when we compared cases to control tissue. Tick-bite associated pathways consisted of keratinization and regulation

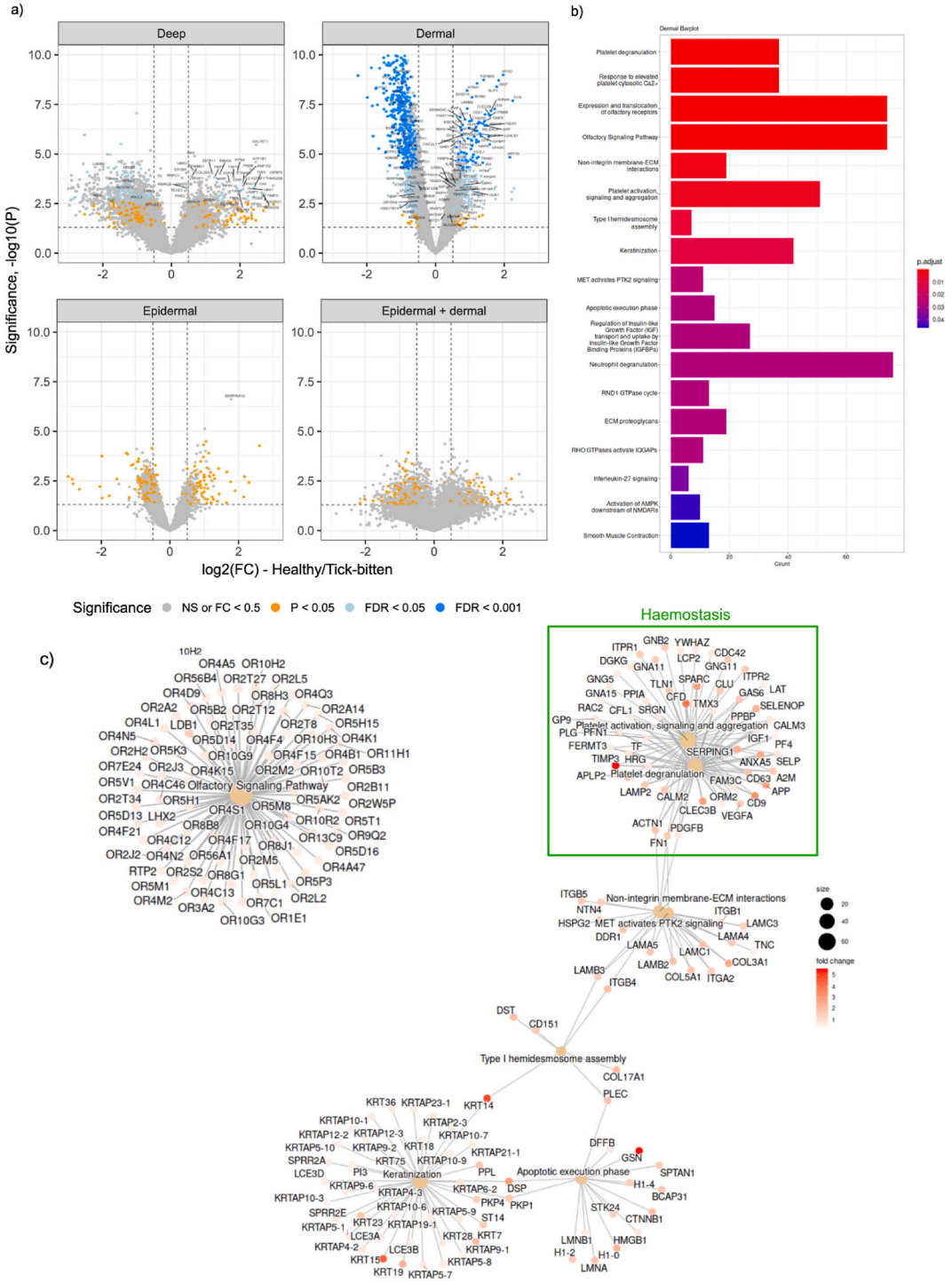

Fig. 6. The dermis identified as the most transcriptionally active site during tick bites. a) DEGs in the different skin locations identified with *P*-value <0.05 and fold change of 1.5 in either direction. Grey: Not significant or under a log2(FC) < 0.5; Yellow: *P*-value<0.05; Cyan: False discovery rate adjusted *P*-value<0.05; Blue: False discovery rate adjusted *P*-value<0.001; b) Bar plot of the significant pathways in the dermal location ranked according to significance; c) Functional enrichment plot of pathways in the dermal region, with haemostasis pathways (green box) identified as most significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

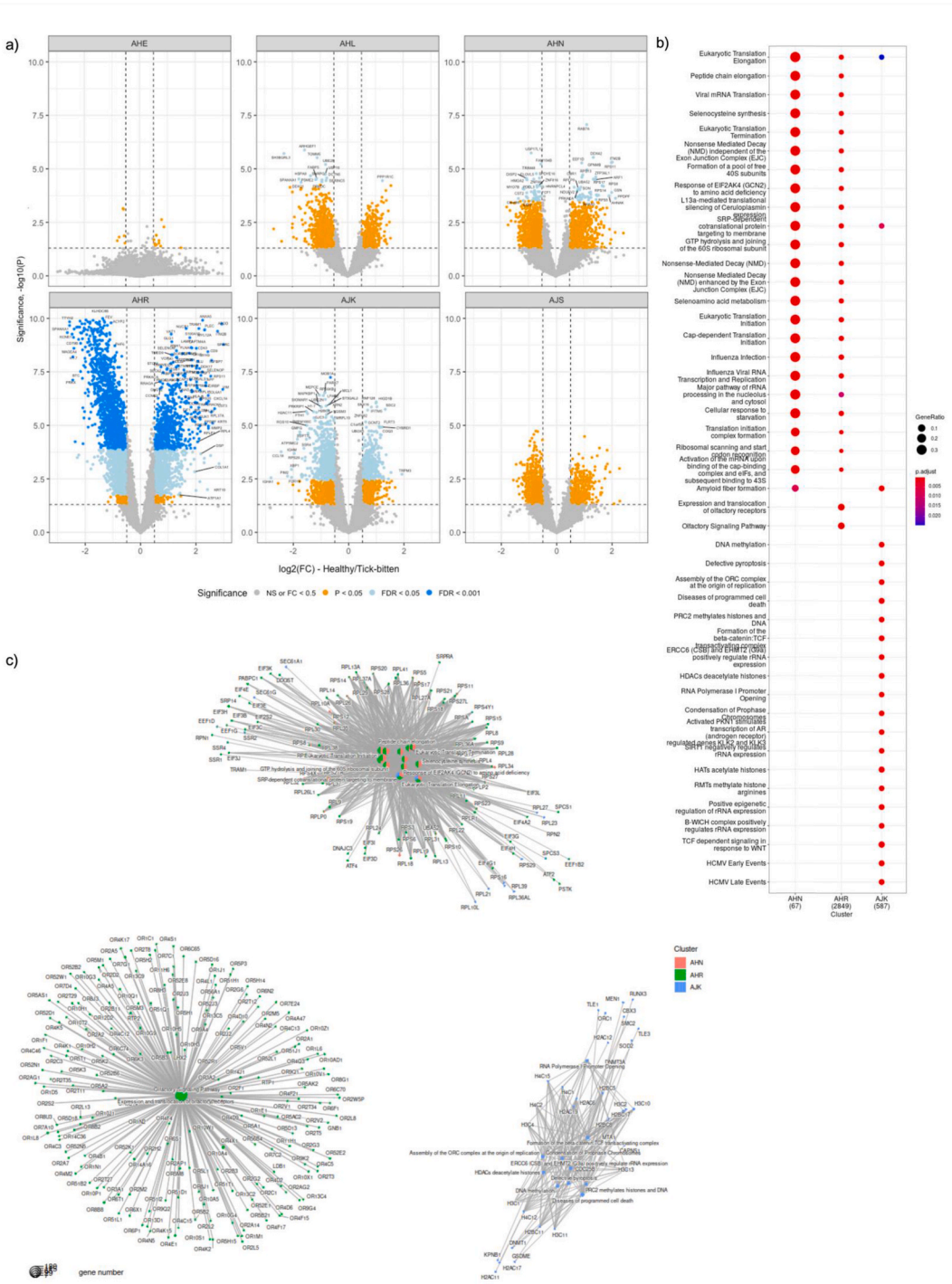

Fig. 7. Acute vs non-acute participants display strikingly different gene expression patterns. Tick bite induced DEGs for each participant; a) Volcano plot of intra-individual DEGs. Grey: Not significant or under a log2(FC) < 0.5; Yellow: *P*-value<0.05; Cyan: False discovery rate adjusted *P*-value<0.05; Blue: False discovery rate adjusted *P*-value<0.001; b) Dot plot of the enriched pathways show similarity between signatures of acute participants differ from that of the chronic participant; c) Functional enrichment plot of significant pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of immune system signals. While recent studies suggest that the interactions between host-specific immune responses and tick species-mediated immune modulation in the tick bitten skin can direct the clinical trajectory of tick associated diseases, the molecular mechanisms involved remain elusive [13–18]. The spatial profiling performed in this study demonstrated an influx of immune cells to

the tick bite sites. Importantly, the change in cellular composition in acutely-bitten participants was similar and included CD8 naïve T cells and naïve B cells, while the chronic participant's tick-bitten section demonstrated an influx of CD4 memory T cells, T-reg cells and a larger magnitude of naïve B cells.

Despite the prominence of microbes in the current understanding of TBD worldwide, only three tick-bitten skin sections in our study had detectable TOI. Rickettsiaceae DNA was present in one sample and *Rickettsia* in the other two samples. In contrast, since *Rickettsia* spp. and *Francisella* spp. are endosymbionts of *A. triguttatum*, it was not unexpected that these TOI were detected in all the ticks tested, indicating that the bite of a *Rickettsia*- or *Francisella*-positive tick does not necessarily result in the detectable transmission of these genera into human skin. All three Rickettsiaceae/*Rickettsia* DNA-positive participants had a history of previous tick bites, yet only one (AHL) had evidence of seroconversion to rickettsial infection. Samples taken from this person one week after tick bite were also positive for dsRNA staining when cultured on vertebrate cells, suggestive of a viral infection. The spatial profiling showed these three participants had a higher proportion of mast cells in some ROIs independent of state, which could be due to an underlying variation in baseline immune tone, hypersensitivity type reaction or host specific responses that spread systemically and influenced the control skin sample. Regardless of the reason for increased mast cell presence in both tick-bitten and control sections, this systemic variation present in both tick-bitten and control biopsies will not be detected by the DE analysis conducted. These data suggests that the clinically heterogenous host responses to tick bites appear influenced not only by variation in introduction of tick-borne material, but the local cellular and molecular host response.

Differentially expressed gene analyses of skin tissues revealed heterogenous responses to a tick bite among participants. Out of the six participants with paired biopsies for within-individual comparison, only four had DEGs that were significantly expressed when comparing tick-bitten and control tissue. Enriched pathway analysis identified pathways for three of the participants with DEGs. G-coupled protein receptors, part of the olfactory signalling pathways, were unexpectedly identified as significant with 74 DEGs in the tick bitten dermis (Fig. 6). Several studies have reported olfactory receptors in non-olfactory tissues and identified a role of these receptors in skin physiology [24–26]. The differential expression of the genes during a tick bite might represent a deviation from healthy physiological processes, indicating a potential disruption in skin homeostasis. Despite individual differences, we were able to discern a pattern of enriched pathways for acute participants, such as pathways associated with metabolism of proteins and viral infection that were distinct to the chronic participant. Conversely, it was interesting to note that the chronic participant had a different pathway signature compared with the acute participants, and instead had epigenetic regulation of gene expression pathways perturbed. Epigenome wide methylation-mediated DNA regulatory mechanisms have been demonstrated to modify biological phenotypes on a cellular level and affect the outcome of complex, chronic diseases such as multiple sclerosis, myalgic encephalomyelitis/chronic fatigue syndrome, fibromyalgia and rheumatoid arthritis [27,28,28–31], conditions that share similar symptomology with PTLDs and DSCATT [32,33]. Perhaps, these epigenetic pathways relate to mechanisms leading to lingering post-tick bite symptoms.

Our study has several limitations. The investigation was designed to account for between-individual variations by utilising skin from a tick-bitten individual's contralateral side as a control. While this approach effectively eliminated variations introduced by patient-specific baselines, the controls could, arguably, be considered not truly 'healthy' as they were obtained from tick bitten participants and could therefore be influenced by systemic responses. Hence, while the within-individual analysis was well-equipped to identify perturbations at the local tick bite site, it was not intended to identify systemic responses. Four of the participants estimated that the tick had been attached to them for more than 24 h, two between 6 and 24 h, and for one the duration of tick bite was unknown. In all cases where the tick was available for analysis, the species was identified as the ornate kangaroo tick (*Amblyomma triguttatum*), which is commonly encountered by bushwalkers and people visiting outdoor areas frequented by kangaroos and other marsupials [34]. In Australia, the ornate kangaroo tick can harbour several pathogens, including *Rickettsia australis* (causative agent of Queensland tick typhus), *Rickettsia gravesii*, and *Coxiella burnetii* (causative agent of Q-fever) [35]. This consideration of participant welfare is ethical but limits the window at which pathogens harboured by the ticks can get inoculated. Furthermore, the tick incriminated in all bites reported here is a species enzootic to only Australia, and therefore extrapolation of these results to outcomes from bites of other species of tick in Australia and globally is not possible. Other limitations include the uneven sex ratio of the main cohort and the relatively small number of participants enrolled and as such causal inferences are only tentative, as the limited number of participants reduces our ability to control for confounding variables.

This technical pilot aimed to assess feasibility, not to provide conclusive mechanistic or biological insight. Not only do our data clearly demonstrate feasibility, it also raises the hypothesis that while TBPs are important causative agents of TBDs, there are other contributing factors such as host-specific factors both at baseline or in response to the tick bite that affect the clinical outcome [13]. In recognizing that analysis of skin biopsies using spatial genomics is more involved for both patient and provider, a possible future direction could investigate systemic signatures obtained from the blood to uncover biomarkers reflecting the local (skin) events. Our current study serves as technical proof of concept that local signatures obtained from the skin can yield signals that differentiate between tick bitten and contralateral control skin samples as well signals that may allow assessment of the role of temporal changes in determining clinical outcome.

4. Materials and methods

4.1. Study design

Adults bitten by ticks within the preceding 72 h in the southwest of Western Australia were enrolled at medical practices during a nationwide tick research study [36]. This study was reviewed by the Murdoch University Human Research Ethics Committee (Permit No. 2019/124) and all samples were de-identified prior to analysis. Exclusion criteria included persons aged under 18 years,

pregnancy, a bleeding tendency (i.e., blood thinning medications, haemophilia, etc), and/or a previous diagnosis of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome or Lyme disease-like illness, recently renamed in Australia as 'Debilitating Symptom Complexes attributed to Ticks', DSCATT [37]. Eligible participants were required to provide written informed consent prior to enrolment and collection of blood samples and skin biopsies. For each participant, 4 mm paired skin biopsies were obtained, one from the tick bite site and one from the contralateral (control) site. Additionally, the biting tick itself and venous blood collected into EDTA tubes were analysed for TBPs using metagenomics and for routine clinical pathology testing as previously described by Barbosa et al. (2022).

4.2. Tick identification and pathogen screening

Biting ticks were identified using morphological methods [36]. Pathogen screening was performed on blood, skin biopsies and biting tick samples obtained from each patient, as previously described [36]. In addition, attempts were made to culture potential bacterial pathogens from the buffy coat (extracted from EDTA blood) and half of the skin punch biopsy, in a range of cell lines that include ISE6 (tick), XTC-2 (amphibian), Ju56 (marsupial), and Vero (mammalian) [36,38,39]. Genomic DNA extracts from the cultures' supernatant, EDTA blood, tissue, and tick samples were screened for bacteria using a high-throughput bacterial 16S rRNA metabarcoding approach on the Illumina MiSeq platform [36,40], with downstream reference to bacterial genera known to be tick-associated, TOI i.e. *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia, Francisella*, *Midichloria*, haemotropic *Mycoplasma* and *Rickettsia* [40]. Additionally, for the detection of tick-associated viruses, serology was performed against known tick-borne viruses that can infect vertebrates in Australia, including Catch-me-cave virus (Phenuiviridae), Gadgets Gully virus (Flaviviridae), Saumarez Reef virus (Flaviviridae), and Finch Creek virus (Nairoviridae). Cultured samples were also assessed for the presence of RNA viruses by probing inoculated cell monolayers with monoclonal antibodies that detect viral dsRNA intermediates in cells (MAVRIC) [41].

4.3. Skin tissue collection, processing, and histology

Skin biopsies were performed using 4 mm dermal punches (Kai Medical, Seki City, Japan) and placed individually in labelled specimen containers pre-filled with 0.9 % sterile saline solution. Each skin biopsy was further bisected vertically with a number 11 scalpel (Livingstone International, Mascot, NSW, Australia) and one half (comprising the epidermis, dermis and subcutaneous adipose layer) was fixed in 10 % formalin, embedded in paraffin blocks and sectioned at a thickness of 5 μm. The other half was frozen in −80 °C in 0.9 % sterile saline for culture and bacterial 16S rRNA amplicon sequencing. A representative set of 5 μm sections were stained with haematoxylin and eosin (H&E), and morphological features were annotated.

4.4. Spatial transcriptomics

For sections assigned to spatial transcriptomic analysis, slides were processed and collected according to manufacturer's instructions (MAN-10151). Briefly, slides were processed for deparaffinisation and epitope retrieval using the automated LEICA BOND Rx system (Leica Biosystems, Wetzlar, Germany). Subsequently, tissue sections were treated with oligo-labelled RNA hybridisation probes from the GeoMx Human Whole Transcriptome Atlas (HuWTA) Human RNA for Illumina Systems (NanoString, Seattle, WA, USA) for 22 h s at 37 °C. Following hybridisation, tissue sections were blocked prior to morphology staining using a mouse anti-human CD3 monoclonal antibody at 1.0 mg/ml (Origene, Rockville, MD, USA) labelled with AF647 (dilution: 1:100) and the proprietary antibodies included in the GeoMx Solid Tumor TME Morphology Kit (NanoString, Seattle, WA, USA) including PanCK labelled with AF532 (dilution: 1:40), and CD45 labelled with AF592 (dilution: 1:40). A SYTO13 nuclear stain (dilution 1:10) was also used. Immunofluorescent morphology makers were used as a guide for the selection of regions of interest (ROIs) encompassing ~200 cell/ROI in the epidermis, dermis and deep dermis (in this study defined as dermis adjacent to subcutaneous adipose tissue). Anatomically comparable areas were selected in the different locations within the skin sections for comparison. For each ROI, UV-cleavable oligo-labels were collected from tissue-bound hybridisation probes for transcriptomic analysis on the NextSeq 2000 Illumina Platform (Illumina, San Diego, CA, USA) (Fig. 1). A total of 95 ROIs were collected for analysis.

4.5. Data analysis

All data analysis was carried out using R v4.3.1 [42] on the raw DCC files obtained after sequencing. Spatial biology quality control and data analysis was carried out with NanoString-validated packages for R, namely GeoMxWorkflows (v1.6.0) [43], GeoMxTools (v 3.5.0) [44,45] and SpatialDecon (v1.10.0) [46]. Differentially expressed genes (DEGs) were determined as genes with an observed fold change of 1.5 in either direction using a linear mixed-effect model (LMM) with a random slope using a Benjamini-Hochberg false discovery rate correction of 0.05 with an adjusted p-value of 0.05, when compared to controls. Visualisation was achieved using pheatmap (v1.0.12) [47] and ggplot2 (v3.4.2) [48]. Pathway enrichment analysis of the DEGs were conducted using ReactomePA (v1.44.0) [49]. Bioinformatics analysis of bacterial 16S rRNA profiling data was performed using established pipelines [36,40].

CRediT authorship contribution statement

Wenna Lee: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rym Ben-Othman:** Writing – review & editing, Conceptualization. **Patrycja Skut:** Writing – review & editing,

Conceptualization. **Amy Huey-Yi Lee:** Writing – review & editing, Supervision. **Amanda D. Barbosa:** Writing – review & editing, Supervision, Project administration, Formal analysis. **Miles Beaman:** Writing – review & editing, Supervision, Funding acquisition. **Andrew Currie:** Writing – review & editing, Supervision. **Nathan T. Harvey:** Writing – review & editing, Formal analysis. **Prasad Kumarasinghe:** Writing – review & editing, Methodology, Formal analysis. **Roy A. Hall:** Writing – review & editing, Funding acquisition. **James Potter:** Writing – review & editing, Formal analysis. **Stephen Graves:** Writing – review & editing, Methodology, Funding acquisition. **Nicholas P. West:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Amanda J. Cox:** Writing – review & editing. **Peter J. Irwin:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Tobias R. Kollmann:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Charlotte L. Oskam:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Peter Irwin reports financial support was provided by National Health and Medical Research Council. The authors declare no competing interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33600.

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