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Microwave therapy for cutaneous human papilloma virus infection

Background: Human papilloma virus (HPV) infects keratinocytes of the skin and mucous membranes, and is associated with the induction of cutaneous warts and malignancy. Warts can induce significant morbidity and disability but most therapies, including cryotherapy, laser, and radiofrequency devices show low efficacy and induce discomfort through tissue destruction. Microwaves are readily capable of passing through highly keratinised skin to deliver energy and induce heating of the tissue in a highly controllable, uniform manner. *Objectives*: To determine the effects of microwave on cutaneous HPV infection. *Materials & methods*: We undertook a pilot study of microwave therapy to the skin in 32 consecutive individuals with 52 recalcitrant long-lived viral cutaneous warts. Additionally, we undertook a molecular characterisation of the effects of microwaves on the skin. *Results*: Tissue inflammation was minimal, but 75.9% of lesions cleared which compares favourably with previous studies showing a clearance rate of 23-33% for cryotherapy or salicylic acid. We show that microwaves specifically induce dendritic cell cross-presentation of HPV antigen to CD8+ T cells and suggest that IL-6 may be important for DC IRF1 and IRF4 modulation to enhance this process. *Conclusion:* Keratinocyte-skin dendritic cell cross-talk is integral to host defence against HPV infections, and this pilot study supports the concept of microwave induction of anti-HPV immunity which offers a promising approach for treatment of HPV-induced viral warts and potentially HPV-related cancers.

Key words: warts, microwave, CD8+ T cells, HPV

Cutaneous HPV infection is common and warts are thought to affect most people at some time during their lives. Point prevalence estimates range from 0.8% to 4.7% of the population and two million people seek medical advice about warts each year in the UK [1], yet treatment options are poor and a meta-analysis has shown no significant benefit over placebo [2]. Although skin is most frequently infected by "non-oncogenic" HPV, most HPV-associated skin squamous cell carcinomas are diagnosed in persistent and recalcitrant verrucae and the majority contain HPV16 [3].

HPV infects the basal epithelial cells of cutaneous and mucosal keratinised epithelia and infection is mainly controlled by T cell-mediated immunity [4]. HPV-specific CD8+ lymphocytes are critical for clearance of HPV viral warts [4] and individuals treated with immunosuppression to prevent organ graft rejection do not clear HPV infections. In healthy individuals, induction of HPV-specific CD8+ T cells with topical imiquimod (TLR7 agonist) has been shown to facilitate wart clearance [5, 6]. However, tissue penetration is a limiting factor for the therapeutic potential of imiquimod on most non-mucosal sites.

Other modalities of thermal ablation have previously been investigated for the treatment of warts [7-10]. Direct heat ablation is now rarely used because of scarring and subsequent morbidity. The most widely used physical modality is liquid nitrogen application (cryotherapy) to the skin [11]. This causes tissue destruction and in a recent meta-analysis of randomised controlled trials, this therapy has been shown to have low efficacy in the management of common warts (with a mean clearance on all sites of 49%) [12]. Microwaves (30 MHz to 30 GHz) exist in the electromagnetic spectrum between radiofrequency and visible light and have been widely used as a means for delivering heat energy to induce thermal ablation in the treatment of cancer, especially for inoperable liver tumours [13], but have not been previously applied to skin. Recent technological advances have enabled development of a hand-held device to deliver targeted application of microwave therapy to skin. We set out to test the potential of this new modality as a treatment for warts in a Phase 1, open-label, uncontrolled clinical study. It was observed in the first few cases that the warts shrank and resolved without obvious necrosis, tissue damage, or inflammation. Hence, we hypothesised that somehow anti-HPV immunity was being activated. We therefore undertook morphological and histological analysis of microwave-treated human skin and investigated for evidence of enhanced anti-HPV immunity. We demonstrated that, even at low energy levels, microwave therapy potentiates cutaneous immunity to HPV.

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Methods and materials

Patients and *in vivo* microwave treatment

The study was approved by the local research ethics committee in accordance with the declaration of Helsinki. Individuals with treatment-refractory plantar warts were recruited. The diagnosis of plantar wart was confirmed by a podiatrist experienced in management of such lesions. A clinically significant wart was defined as >one year duration, with at least two previous failed treatments (salicylic acid, laser, cryotherapy, needling, and surgical excision). Exclusions were pregnancy or breast feeding, pacemaker *in situ*, metal implants within the foot or ankle, co-morbidities affecting immune function, or capacity to heal. At each study visit, a complete examination of the affected area was undertaken and a quantitative measure of pain and neuromuscular function assessed. No dressing was required and volunteers continued normal everyday activities after treatment with no restrictions.

A total of 32 volunteers with 54 foot warts were enrolled into the study (17 males and 15 females; age range: 22-71 years; mean: 44·79 years [SD: 13.019]). Sixteen were solitary and 38 multiple-type warts (*e.g.* mosaic verrucae). Mean lesion duration was 60.54 months (range: 12-252) and diameter 7·43 mm (range: 2-38 mm; SD: 6.021). At the conclusion of the study period, one patient had been lost to follow-up and two patients had withdrawn (n = 3; four warts) but were retained in the statistical analysis, classified as unresolved lesions.

Microwave treatment (Swift®, Emblation Medical Ltd., UK) of the most prominent plantar wart was titrated up, as tolerated to 50 J over a 7-mm diameter application area (130 J/cm^2) over 5 seconds (10 watts for 5 seconds). Lesions >7 mm received multiple applications until the entire surface of the wart had been treated. If the wart persisted, treatment was repeated at one week, one month, three months, and 12 months. Response to treatment was assessed by the same investigator as binary; "resolved" or "unresolved". Resolution was indicated by fulfilling three criteria: (1) lesion no longer visible; (2) return of dermatoglyphics to the affected area; and (3) no pain on lateral compression. Pain was assessed using a 10-point visual analogue scale.

Human skin and blood samples

Skin and blood samples for microwave experiments were acquired from healthy individuals as approved by the local Research Ethics Committee in adherence to Helsinki Guidelines.

Histological analysis

Skin samples were treated immediately *ex-vivo* with microwaves (Swift s800; Emblation Ltd., UK) or liquid nitrogen therapy and punch biopsies taken from treated skin were sent for histological analysis or placed in culture media.

Histological analysis of hematoxylin and eosin (H&E)-stained tissue sections was undertaken following fixation and embedding in paraffin wax. DNA damage was assessed by staining for single-stranded and double-stranded DNA breaks by TUNEL assay using the ApopTag® In Situ Apoptosis Detection Kit (Millipore, UK). Following culture, supernatants were collected and analysed for lactate dehydrogenase (LDH) release using the Cytotoxicity Detection Kit (Roche applied science) as a measure of apoptosis.

Cell culture and *in vitro* microwave treatment

Primary keratinocytes were obtained from pooled neonatal foreskin donors (Lonza, Switzerland) and cultured in keratinocyte growth medium 2 (PromoCell) at 37°C, 5% CO_2, until 70-90% confluency for use in experimental work (P4-P10).

Human skin explant cultures and human HaCaT keratinocytes were cultured in calcium-free DMEM (ThermoFisher Scientific) with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 10% foetal bovine serum (FBS), and supplemented with calcium chloride at 70 μM final concentration.

Microwave treatment of cells in culture was delivered in a flat-bottomed well using the Swift device applied directly to the plastic base from the underside. To assess whether the plastic caused loss of microwave energy in our system, the 150 J Swift programme applied through the culture well base delivered a temperature rise of 18.6°C (SD: 1.1) to 200 g of culture media, equivalent to ~15.61 J (SD: 0.92). Thus, it could be estimated that 15 J applied *ex vivo* would be equivalent to ~150 J as tested here *in vivo*. However, energy loss during skin application would reduce this difference, but calculation of the precise transfer of energy to skin *in vivo* was not possible, so we estimate that the dose delivered *in vitro* is up to 10-fold lower than that by direct skin application *ex vivo*. To avoid confusion, the setting on the Swift system is the energy level referred to throughout the manuscript (in human and *in vitro* studies).

Lymphocytes were cultured in RPMI-1640 medium with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 292 μg/mL L-glutamine, supplemented with 10% FBS or 10% heat-inactivated human serum (HS). HaCaT cells were cultured to sub-confluency to avoid cell differentiation and used in assays at passage 60-70. Cells were plated at 2.5×10^3 cells/well in 96-well flat plates (Corning Costar) and cultured overnight to reach confluence. HaCaTs were washed once with PBS before treatment with microwaves, liquid nitrogen (10 seconds), or with LPS+IFN-γ (1 ng/mL+1,000 U/mL). Cells were cultured for 24 hours before supernatants were harvested. HPV16 E7 protein was expressed in *E. coli* at the Protein Core Facility of Cancer Sciences Unit, University of Southampton. Endotoxin was removed using Detoxi-Gel endotoxin removal using columns (Thermo Scientific).

For HPV-specific T cell lines, PBMCs were isolated from HLA-A2-positive individuals, as previously described [14]. PBMCs were seeded at $2\text{-}4 \times 10^6$ cells/well in 24-well culture plates and 10 μg/mL ninemer HLA-A2-restricted HPV16 epitope LLM (LLMGTLGIV) [15] was added; cells were cultured in 1 mL RPMI+10% HS. On Day 3, cells were fed with RPMI+10% HS+IL-2 (200 IU/mL), and then fed again on Day 7 or when needed. After Day 10, HPV-specific T cells were harvested for cryopreservation before testing against HPV using ELISpot assays.

To generate monocyte-derived dendritic cells (moDCs), CD14+ cells, were positively isolated from PBMCs by

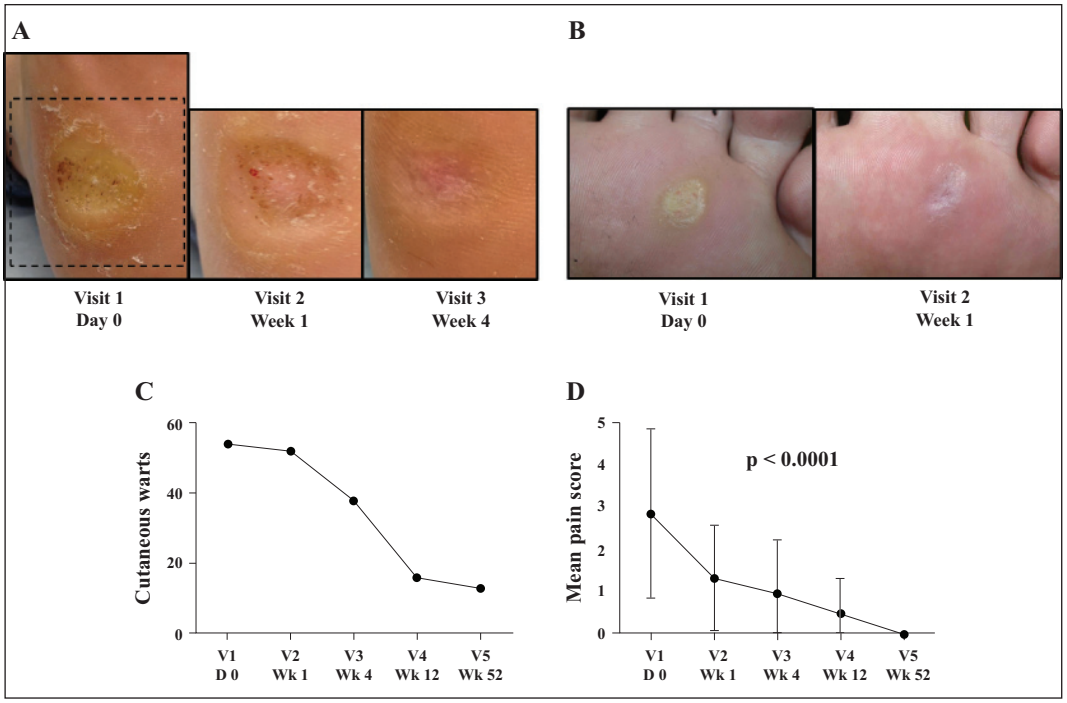

Figure 1. Response of recalcitrant warts to microwave therapy. **A)** Clinical image of plantar wart pre-microwave treatment (left), after one treatment (middle), and after two treatments (right). **B)** Clinical image of plantar wart pre-microwave treatment (left) and after one treatment (right). **C)** Intention to treat analysis of 32 patients with 54 HPV foot warts treated by microwave therapy over five visits: baseline, one week, one month, three months, and 12 months. Resolved warts were enumerated. **D)** Pain scores were assessed using a 10-point visual analogue score at each visit. Statistical test: one-way ANOVA.

magnetic separation using CD14 microbeads (Milentyi Biotec, UK), according to the manufacturer's protocol. Cells were washed and resuspended in RPMI+10% FBS+250 U/mL IL-4 and 500 U/mL GM-CSF. At Day 3, cells were fed with RPMI+10% FBS+IL-4 and GM-CSF, and then harvested on Day 5 for use in functional assays.

ELISpot, flow cytometry and qPCR

Keratinocytes (HaCaTs or primary as indicated) were treated with microwaves at various energy settings before removal of supernatant at various time points. MoDCs were treated overnight with keratinocyte supernatant, then washed twice before incubation with LLM peptide (10 μg/mL for 2 hours) or HPVE7 protein (10 μg/mL for 4 hours) before a further wash. Human IFN-γ ELISpot (Mabtech, Sweden) was undertaken, as per the manufacturer's protocol and as reported previously [14]. moDCs at 1×10^3 were plated with autologous HPV peptide-specific T cells at a ratio of 1:25. Spot forming units (sfu) were enumerated with ELISpot 3.5 reader (AID, Germany).
MoDCs were treated with HaCaT supernatant and harvested at 24 hours for flow cytometric analysis of cell phenotype. Cells were stained with violet LIVE/DEAD stain (Invitrogen, ThermoFisher, UK) for 30 minutes at 4°C, then washed with PBS+1% BSA and stained with antibodies PerCP-Cy5.5 anti-HLA-DR, FITC anti-CD80, FITC anti-CD86, or PE anti-CD40 (Becton Dickinson, UK) for 45 minutes at 4°C. Cells were washed, then resuspended in PBS+1% BSA, and analysed using the BD FACSAria and the FlowJo v10.0.08 analysis software.

The expression of chosen genes was validated with quantitative PCR using the TaqMan gene expression assays for target genes: *YWHAZ* (HS03044281_g1), *IRF1* (Hs00971960_m1), and *IRF4* (Hs01056533_m1) (Applied Biosystems, Life Technologies, UK) in human skin, and treated as indicated. RNA extraction (RNeasy mini kit, Qiagen) and reverse transcription (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems; ThermoFisher Scientific UK)) were carried out accordingly to the manufacturer's protocol.

Results

Treatment of human papilloma virus infection in humans with microwave therapy

Of the 32 volunteers with severe warts, 54 treatment-refractory plantar warts were treated with microwave therapy (*figure 1A, B*). At the end of the study period, of the 54 warts treated, 41 had resolved (75.9%) and nine remained unresolved (16.7%), and two patients (with three warts [5.6%]) withdrew from the study and one patient (with one wart [1.9%]) was lost to follow-up. The mean number of days to resolution was 79.49 days (SD: 34.561; 15-151 days). Of the resolving lesions, 94% had cleared after three treatments (*figure 1C*). No significant difference in resolution rates between males and females (p = 0·693) was observed. Statistically significant reductions in pain were observed as treatment progressed (p<0.0001) (*figure 1D*). Adverse events were minimal. One patient reported

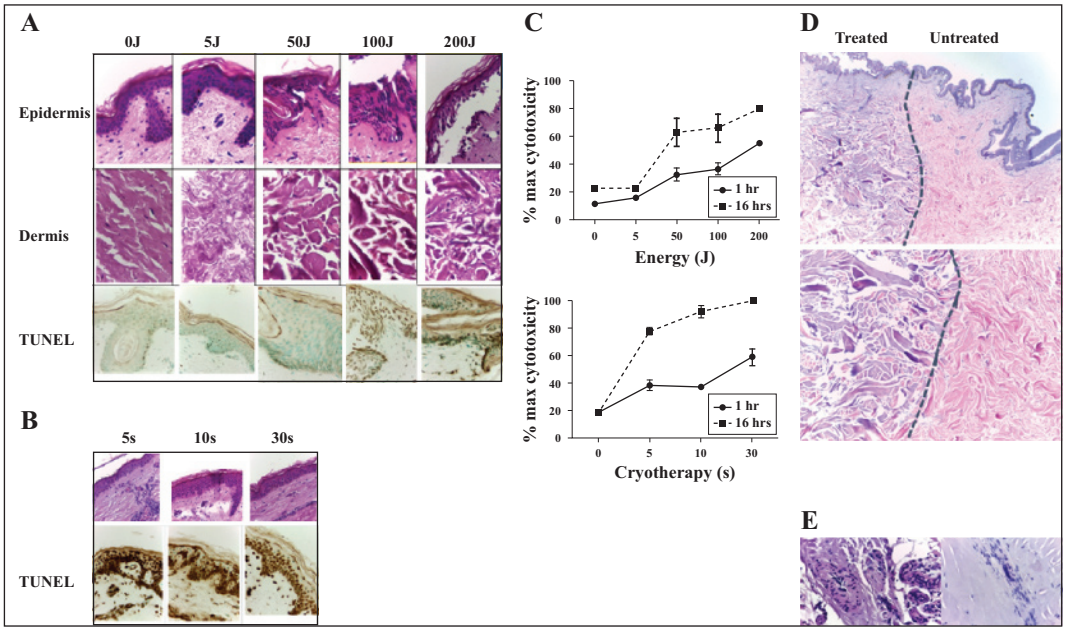

Figure 2. Microwave effects on human skin. **A)** Histological analysis of normal human skin treated with microwave stimulation visualised in the epidermis/papillary dermis (upper and lower panels), or deep dermis (middle panels). Skin was subjected to microwave therapy (0-200 J) before punch excision. Tissue was cultured for one hour before fixation and paraffin embedding (H&E or TUNEL staining; original magnification: ×20). **B)** Histological analysis of human skin treated with liquid nitrogen therapy for 5, 10, or 30 seconds, before punch excision. Tissue was cultured for one hour before fixation and paraffin embedding (H&E or TUNEL staining; original magnification: ×20). **C)** Following microwave therapy (upper panel) or cryotherapy (lower panel), skin samples (in triplicate) were excised and cultured in media for one or 16 hours before measurement of cytotoxicity, assessed by harvesting supernatant to measure supernatant lactate dehydrogenase (LDH) release by ELISA. **D)** Skin was subjected to microwave therapy (150 J) before punch excision at the margin of the treated zone. Tissue was cultured for one hour before fixation and paraffin embedding (H&E stain; original magnification: ×10 [upper panel], x100 [lower panel]. **E)** Skin was subjected to microwave therapy (150 J) before punch excision. Tissue was cultured for one hour before fixation and paraffin embedding (H&E stain showing deep dermis adnexae: glandular [left] and vascular [right]; original magnification: ×100). Data are representative of three independent experiments.

transient pain from the treatment which required a simple oral analgesic (paracetamol) and resolved within 24 hours. This individual withdrew from the study. No further adverse events were reported. No cases of scarring were recorded following completion of treatment. No cases of neuromuscular dysfunction were reported.

Microwave treatment of human skin

Human skin has not previously been reported to be treated with microwave therapy, therefore, we proceeded to undertake a full histological analysis of treated skin. Skin removed during routine surgery was treated *ex vivo* and one hour after treatment punch biopsies were taken and fixed for histological processing. Neither macroscopic nor histological changes were noted with the lowest energy setting (5 J). At 50 J, mild macroscopic epidermal changes only were noted, and microscopically minor architectural changes and slight elongation of keratinocytes were seen without evidence of altered dermal collagen. At higher energies (100/200 J), gross tissue contraction was visible macroscopically. Microscopic changes in the epidermis were prominent, showing spindled keratinocytes with linear nuclear architectural changes and subepidermal clefting (*figure 2A*). Dermal changes were prominent at energies of 100 J and above and showed a homogenous hyalinised zone of papillary dermal collagen, thickened collagenous substances, and accentuation of basophilic tinctorial staining of the dermal collagen with necrotic features (*figure 2A*). These features are similar to electro-cautery artefacts and suggest that at >100 J, there is the potential to coagulate proteins and induce scarring. Histological analysis both at 16 hours and 45 hours showed similar changes (data not shown).

In clinical practice, cryotherapy is delivered to the skin by cryospray, which is time-regulated by the operator. In contrast to microwave therapy, minimal epidermal or dermal architectural change was identified with cryotherapy at standard treatment duration times (5-30 seconds), but did show a dose-dependent clumping of red blood cells in vessels (*figure 2B*).

Tissue release of LDH acts as a biomarker for cellular cytotoxicity and cytolysis. To examine the extent of cell death induced by microwave irradiation, human skin was treated with 0, 50, 100 or 200 J before punch excision of the treated area and incubation in medium for one hour or 16 hours. Measurement of LDH revealed a dose-dependent induction of tissue cytotoxicity with increasing microwave energies (*figure 2C*). In line with the lack of histological evidence of cellular damage, at 5 J, cytotoxicity of microwave application was equivalent to control. Early cytotoxicity was not prominent at 50 J, but became more evident after 16 hours. Higher energy levels induced more prominent cytotoxic damage. In contrast to microwave therapy, liquid nitrogen

treatment of skin induced cytotoxicity at the lowest dose both at one hour and 16 hours.
Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) identifies cells in the late stage of apoptosis. Analysis at 0, 5, 50, 100 and 200 J identified increased cellular apoptosis in the epidermis above 100 J (*figure 2A*). In contrast, cryotherapy induced significant epidermal and dermal DNA fragmentation (*figure 2B*).
The physics of microwave therapy suggests a sharp boundary between treated and untreated tissue with minimal spreading of the treated field. This was borne out histologically by a clear demarcation between treated areas extending vertically from the epidermis through the dermis (*figure 2D*). Examination of the dermis showed that microwave therapy modified skin adnexae, inducing linear nuclear architectural changes in glandular apparatus, micro-thrombi, fragmented fibroblasts, and endothelial cells (*figure 2E*).

Microwave induction of immune responses in skin

We first examined the response of keratinocytes to microwave therapy *in vitro*. In analysing *in vitro* the effects of microwave therapy, it was necessary to apply the microwave treatment through culture dish plastic. Thus, the energy setting *in vitro* is equivalent to a lower energy setting than with direct application *in vivo* (*see above*). In keratinocyte monolayers (HaCaT), apoptosis was induced by microwave therapies above 100 J *in vitro* (*figure 3A*). Only above the apoptotic threshold (100 J) were surface phenotypic changes of cellular activation noted in viable cells with increased expression of HLA-DR, CD40, and CD80 (*figure 3B*). Next, we utilised a model of skin cross-talk of keratinocyte signalling to dermal dendritic cells. Initially, we observed strong activation of MoDCs primed with supernatant from microwave-treated keratinocytes (*data not shown*), but we wished to disentangle the pro-inflammatory effects of apoptosing/necrotic cells from viable cell cross-talk. Therefore, keratinocytes were treated with microwave therapy as above, and washed after eight hours to remove dead or apoptotic cells. Treated keratinocytes were then incubated for a further 16 hours before supernatant collection to prime moDCs, which had not been directly exposed to microwave therapy. The supernatants induced potent induction of moDC activation with increased expression of CD86, CD80, and to a lesser extent, CD40 (*figure 3C*).
We next set out to model the functional outcome on skin dendritic cells following microwave treatment of keratinocytes. Keratinocyte monolayers (HaCaT) were untreated, or microwave- or cryotherapy treated before supernatant harvesting. Supernatant-primed DCs were pulsed with a nine-amino acid HLA-A2 epitope (LLM) from human papilloma virus (HPV) E7 protein and cultured with an autologous HPV-specific CD8+ T cell line. As expected, in all conditions, the moDCs efficiently presented HPV peptide to HPV-specific CD8+ T cells, inducing IFNγ (*figure 4A*). However, dendritic cell presentation of HPV is dependent upon cross-presentation to the MHC class I pathway. Therefore, we also tested the capability of untreated, microwave-treated or cryotherapy-treated KC-primed moDCs to present HPV E7 protein to an HLA-matched HPV-specific CD8+ T cell line. Strikingly, only microwave-treated KCs were capable of priming moDCs to enhance cross-presentation (*figure 4B*). To explore the potential mechanism of keratinocyte response to microwave therapy, we confirmed up-regulation of HSP-70 in response to microwave therapy of keratinocytes (*figure 4C*). Although, the assay used did not distinguish constitutive from inducible HSP-70, we clearly demonstrated global increase in HSP-70 expression following microwave therapy. Additionally, IL-6, but not IL-1β or TNF-α, was expressed in response to microwave stimulation, which suggests that alternative inflammatory signalling pathways from that seen in cryotherapy-treated cells are induced by microwave stimulation (*figure 4D*). To further explore the potential innate immune signalling pathways in keratinocytes following microwave therapy, we examined IRF1 and IRF4. These transcription factors are key regulators of dendritic cell activation of adaptive immunity. We show that microwave therapy induced down-regulation of IRF1 and up-regulation of IRF4 (*figure 4E*).

Discussion

This is the first study to investigate the potential efficacy of locally delivered microwaves in the treatment of cutaneous viral warts. In this uncontrolled pilot study, we report a complete resolution rate of 75.9% of recalcitrant plantar warts (with an average lesion duration of over five years). This compares very well with previous reports of plantar wart resolution for salicylic acid and or cryotherapy (23-33%) [16].
For all novel therapies, adverse events are critical but we did not identify a strong signal for adverse events. As with current physical treatments for warts, discomfort is expected for the patient. During the study, patients generally reported that for a typical five-second treatment, they endured moderate discomfort for approximately two seconds, which immediately diminished after the treatment had completed. In addition, it was commonly noted that discomfort was less with subsequent treatments. One male patient withdrew from the study after one treatment, citing the pain of treatment as the reason. In the study design phase, pre-operative use of topical anaesthetic cream was tested, but appeared to do little to mitigate the pain (*unpublished data*) and it was felt that the pain of local anaesthetic injection would exceed that normally experienced during a microwave treatment. Following microwave therapy, patients did not require dressings or special advice as no wound or ulcer was caused, allowing the patient to continue normal activity. The short microwave treatment time (five seconds) offers a significant clinical advantage over current wart therapies, such as cryotherapy and electro-surgery. Within five seconds, microwaves penetrate to a depth of over 3.5 mm at the energy levels adopted for the study [17]; possibly a greater depth than can be attained by cryosurgery or laser energy devices. Moreover, microwaves, like all forms of electro-magnetic radiation, travel in straight lines and energy is deposited in alignment with the "beam" emitted from the device tip with little lateral spread, meaning minimal damage to surrounding tissue, as confirmed in this study. Microwaves induce dielectric heating. When water, a polar molecule, is exposed to microwave energy, the

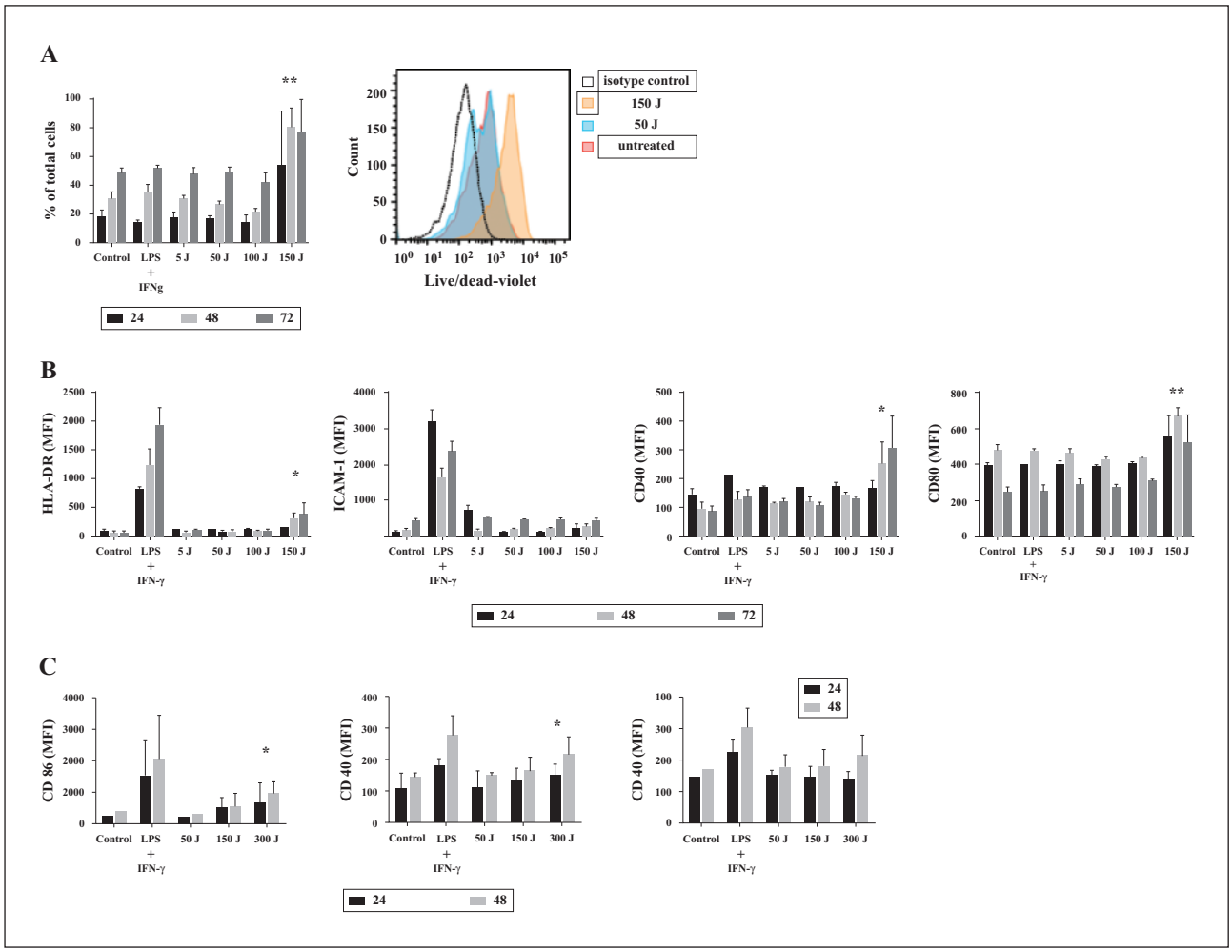

Figure 3. Microwave activation of keratinocytes and dendritic cells. **A)** Left: flow cytometric analysis of viable keratinocytes (% of total cells) indicated by negative staining with the amine reactive viability dye LIVE/DEAD after control, microwave (5-150 J), or LPS/IFN-γ treatment. Keratinocytes were treated then kept in culture for 24 (black bars), 48 (light grey) or 72 (dark grey) hours before analysis. Right: flow cytometric analysis of keratinocyte viability after microwave therapy or control, depicted as a histogram. X-axis: LIVE/DEAD stain; y-axis: cell count. **B)** Flow cytometric analysis of HLA-DR, ICAM-1, CD40 or CD80 expression on viable keratinocytes. Keratinocytes were treated with microwave therapy (5-150 J), LPS/IFN-γ, or nil (control), rested in culture for 24 (black bars), 48 (light grey) or 72 (dark grey) hours, before analysis of the viable population. **C)** Flow cytometric analysis of CD86, CD80, and CD40 expression on viable monocyte-derived dendritic cells (moDCs). Keratinocytes were treated with microwaves (5-150 J), LPS/IFN-γ, or untreated (control), rested in culture for eight hours, then washed. They were left in culture for the remaining time until 24 (black bars) or 48 (light grey) hours, before transfer of supernatant onto moDCs. MoDCs were incubated for 24 hours before harvesting for analysis. Data are representative of three independent experiments. Mean+SD; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

molecule is excited and rotates to align with the alternating electro-magnetic field. At microwave frequencies, the molecule is unable to align fully with the continuously shifting field resulting in heat generation. Within tissues, this acts to rapidly elevate temperatures. This process increases cellular temperature because it does not depend on tissue conduction. Microwave treatment produces no vapour or smoke unlike ablative lasers and electro-surgery, eliminating the need for air extraction systems due to the risk of spreading viral particles within the plume [18].

Although microwave therapy has been considered a tissue ablation tool, we observed minimal skin damage after treatment with 50 J, yet good clinical responses were seen. Therefore, we investigated whether there was evidence to support an induction of anti-HPV immunity by microwave therapy. The critical nature of CD8+ T cell immunity for host defence against HPV skin infection is well established and supported by the observation of increased prevalence of infection in immunosuppressed organ-transplant recipients [19], and that induction of protection from HPV vaccines is mediated by CD8+ T cells [20]. We show here that microwave therapy of skin induces keratinocyte activation and cell death through apoptosis. However, *in vitro* microwave-primed keratinocytes are capable of signalling to dendritic cells and enhancing cross-presentation of HPV antigens to CD8+ lymphocytes at microwave energy levels equivalent to or lower than that used in the clinical study, which offers a potential explanation for the observed response rate in our clinical study. *In vitro* evidence suggests that this is likely

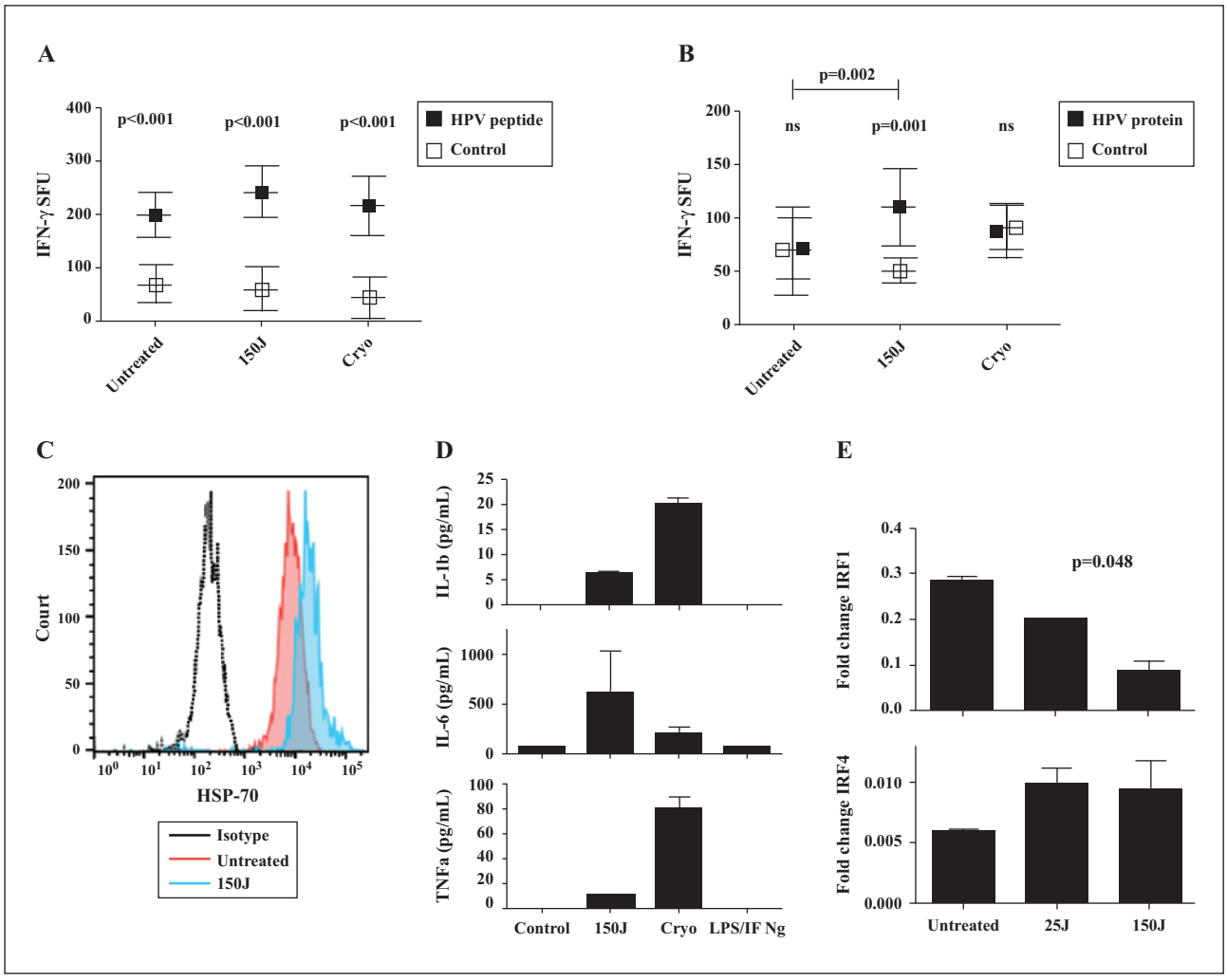

Figure 4. Microwave induction of HPV antigen cross-presentation. **A)** ELISpot assay of IFN-γ production by HPV-specific CD8+ cells following co-culture with HPV peptide-pulsed moDCs primed by supernatant from untreated, microwave-treated (150 J) or cryotherapy (cryo)-treated HaCaT keratinocytes. MoDCs were primed with supernatant for 24 hours prior to pulsing with control (open squares) or HPV peptide (closed squares) for two hours. Pulsed moDCs were then co-cultured with an HLA-matched CD8+ HPV-specific T cell line before assay with IFN-γ ELISpot. Statistical significance was determined using the Holm-Sidak method, with alpha = 5%. Data are representative of three independent experiments (mean+SD). **B)** ELISpot assay of IFN-γ production by HPV-specific CD8+ cells following co-culture with HPV E16 protein-pulsed moDCs primed by supernatant from untreated, microwave-treated (150 J) or cryotherapy (cryo)-treated HaCaT keratinocytes. MoDCs were primed with supernatant for 24 hours prior to pulsing with control (open squares) or HPV protein (closed squares) for two hours. Pulsed moDCs were then co-cultured with an HLA-matched CD8+ HPV-specific T cell line before assay with IFN-γ ELISpot. Statistical significance was determined using the Holm-Sidak method, with alpha = 5%. Data are representative of three independent experiments (mean+SD). **C)** Flow cytometric analysis of intracellular HSP-70 expression on viable keratinocytes after microwave therapy or control, depicted as a histogram. Primary human keratinocytes were treated with microwave therapy (150 J), or nil (untreated), rested in culture for 24 hours, before analysis. X-axis: anti-HSP-70; y-axis: cell count. **D)** ELISA of IL-6, TNFα, and IL-1β production by primary human keratinocytes 24 hours after treatment with microwave therapy (150 J), LPS/IFNg, cryotherapy or control (untreated). **E)** Fold expression of change of IRF1 and IRF4 over a housekeeping gene in normal human skin with microwave therapy (25 J and 150 J) by qPCR.

to be mediated by cross-talk between microwave-treated skin keratinocytes and dendritic cells, through induction of danger-associated molecular patterns (DAMPs), such as HSP-70 in keratinocytes, resulting in up-regulation of DC CD40 and CD80/86 and subsequent enhanced cross-presentation of HPV proteins to CD8+ T cells. Microwave therapy also specifically induced enhanced IL-6 synthesis from keratinocytes. IL-6, is a pro-inflammatory mediator, important in anti-viral immunity, which has been recently shown to induce rapid effector function in CD8+ cells [21]. Thus, IL-6 up-regulation may provide an important additional mechanism for microwave-induced anti-viral immunity. The intriguing contrast between cryotherapy and microwave therapy revealed a far greater release of IL-1β and TNF-α with cryotherapy which, in addition to the lesser IL-6 induction, may offer potential to utilise the treatments for different situations where IL-1β/TNF-α-driven inflammation may be preferable, or vice versa.

Additionally, the specificity of inflammatory pathways induced by each modality may explain why cryotherapy and microwave stimulation may not show equal effectiveness in the same disease.

IRFs have been shown to be central to the regulation of immune responses [22-24]. IRF4 is essential for differentiation of cytotoxic CD8+ T cells [25, 26], but up-regulation in dendritic cells has also been shown to enhance CD4+ differentiation [23], therefore, this pathway may potentially enhance both CD8+ immunity and T-cell help following microwave treatment. IRF1 expression has been previously reported to be modulated by HPV infection, but different models have shown opposite outcomes [27, 28]. We show down-regulation of IRF1 in human skin in association with a microwave therapy, which supports the proposal of IRF-1 as a therapeutic target in HPV infection [28].

This study is the first of its kind to study microwaves in the treatment of plantar warts *in vivo*. Further work to examine the immune infiltrate in microwave-treated warts is planned. Whilst we acknowledge the limitations of the uncontrolled, non-randomised design, the promising results shown here suggest that a randomised controlled study with a larger sample size is warranted to confirm the efficacy of this treatment. ■

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Case Reports in Dermatology

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Single Case

The Successful Use of a Novel Microwave Device in the Treatment of a Plantar Wart

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Keywords
Warts · Verrucae · Treatment · Infections · Microwave · Human papilloma virus

Abstract
Plantar warts, caused by the human papilloma virus (HPV), are a commonly encountered condition presenting in clinic. In adults, an array of various therapies exists, frequently with modest results particularly with plantar lesions. Microwaves have had limited uses for medical purposes. Recently a new portable microwave device has been approved for the treatment of skin lesions. Prior research has demonstrated immuno-stimulatory effects against HPV infection. We report the application of a novel portable medical microwave unit to treat a long-standing plantar wart which had failed to respond to other treatment modalities.

Introduction

Cutaneous warts are a common clinical problem estimated to affect between 7 and 12% of the population [1]. For many younger patients, natural resolution is a common feature, but in adults they often remain stubborn and refractory to treatment. As highlighted in re-

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cent guidelines, long-term persistence and failure to respond to therapy are more commonly features of cutaneous warts located on the plantar area of the foot [2]. In addition to the low success rate of common therapies such as salicylic acid and cryosurgery for warts in this location, these therapies cause local irritation and pain making their use unfavourable [2].

Case Report

A 41-year-old male with a stubborn, single plantar wart on his right styloid process which had been present for over a year and had failed to respond to treatment with cryosurgery (Fig. 1). The lesion was described as painful when standing. The lesion measured 10 by 10 mm, and the patient rated the pain level as an 8 out of 10 (numeric rating scale) at the initial assessment. He had no significant past medical or medication history. Following a discussion with the patient about the various options available, the patient consented for treatment using microwave energy. After gentle reduction of the hyperkeratotic skin overlying the lesion, microwave energy was applied using the Swift S800 Microwave Device (Emblation Medical Ltd, Alloa, UK). Device settings were programmed to deliver 50 J (10 W for 5 s). Microwaves were delivered without topical or local anaesthetic to the verruca through an applicator applied directly onto the surface of the lesion. After intervention, no dressing was required, and the patient was able to mobilise freely. After 3 weeks, the patient was reviewed. The treated area demonstrated large amounts of bruising (Fig. 2). Pain measurement was recorded as 2 out of 10 (NRS), corresponding to a 75% improvement compared to pre-operative levels. Interestingly, the patient noted that pain had reduced almost immediately following the first microwave ablation. A second treatment of 50 J was administered to the same area under the same settings. Two weeks later, the lesion was almost completely resolved (Fig. 3) and pain score assessment was 0 out of 10 (NRS). At 6-month follow-up, the lesion remained fully resolved (Fig. 4).

Discussion

This case represents a report of successful treatment of a cutaneous wart using a portable microwave therapy device. The device was developed and subsequently "CE" marked as a medical device for the general indications "in the treatment and ablation of skin lesions." In this singular case presentation, the microwave device proved successful and creates a pathway for further research into the treatment of plantar warts. Microwaves are a type of non-ionising radiation in the 300 MHz to 300 GHz wavelength range of the electro-magnetic spectrum. Application to the skin works on the principle of dielectric heating. Polar molecules, such as water, when exposed to microwave energy, rotate and attempt to align with the changing electro-magnetic field. At microwave frequencies, rapidly rotating molecules generate heat which is dissipated to the surrounding tissues and acts to rapidly elevate temperatures. To date, the medical applications of microwaves have been relatively limited and not widely explored. The technology has been successfully utilised intra-operatively to ablate large tumours [3, 4] but little work has focused on its applications in the treatment of skin lesions. The unit produces microwave energy within the 8 GHz range from an internal generator, delivered through a cable into a ceramic cap, directly into the tissues to which it is applied. The applicator tip of the device is single use reducing the risk of cross-infection. The physical properties of microwaves potentially offer significant advantages over cryotherapy

and laser modalities when used medically on the skin. Firstly, as microwaves travel through tissue in straight lines in alignment with the device tip, there is minimal lateral spread of heat, meaning minimal damage to surrounding tissue. Moreover, microwave energy is not dependent on tissue conductivity, and tissue effects can therefore be produced much more rapidly than with cryotherapy cooling. Compared to laser light, microwaves are not modified by chromophores, and therefore microwaves show a greater depth of penetration into the skin. At powers of up to 10 W, a penetration of up to 3 mm can be expected. An additional advantage is that the procedure does not induce vapour, smoke or particulate debris, which is a common problem with high energy lasers, and has been reported to spread viral particles within the plume [5]. Additionally, the device causes no skin breakage at site of application (at this energy setting) and so did not require any post-operative dressing, allowing the patient continue normally activity. The benefits of a heat therapy over a cold treatment such as liquid nitrogen application can be seen as the wart virus, although stable in temperatures of 196° C is more sensitive to heating than cold.

Studies on the effects of heating normal and human papilloma virus (HPV)-infected tissue suggest that it may promote the induction of adaptive immunity [6–10]. Microwave damage to HPV in an in vitro study on genital warts was compared with that caused by treatment with a CO_2 laser. The authors of the work concluded that microwaves were significantly more effective at denaturing HPV than the comparative device [11]. Preliminary studies of human skin explants exposed to low-level microwave energy suggested that increased danger signalling in keratinocytes, including induction of HSP70, may be the critical pathway for cutaneous wart resolution. Microwave-treated keratinocytes were able to induce dendritic cell activation (CD80, CD86, CD40) and enhancement of anti-HPV responses by CD8+ T cells [12, 13]. This case study represents an exciting additional therapeutic option for treatment of recalcitrant cutaneous warts. We acknowledge the limitations of reporting a single case, and further work to address this in a randomised controlled trial setting is underway.

Conclusion

This work represents a single case study of a persistent and painful wart treated successfully using microwave energy applied directly to the lesion using a novel medical device. Further work is required to fully assess the effectiveness of this therapy.

Statement of Ethics

This case study was drawn from an ongoing project which has been granted full ethical approval from the Faculty of Health Sciences, University of Southampton. Full consent was obtained from the patient for use of this case study.

Disclosure Statement

I.R.B. is a consultant for Emblation Medical Limited.

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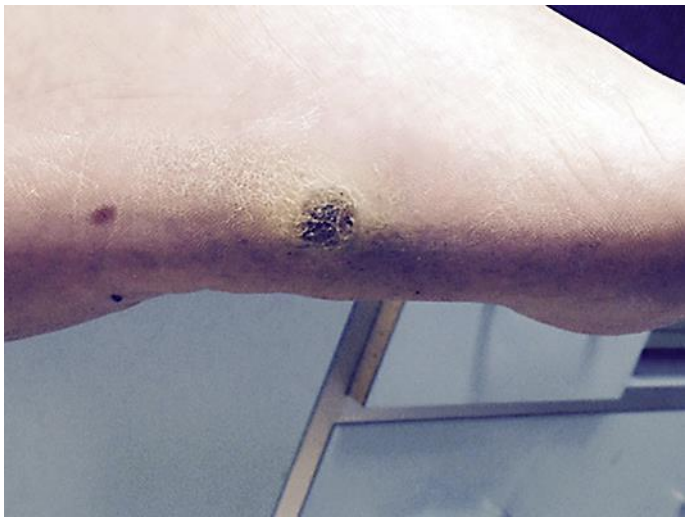


Fig. 1. Lesion at presentation (10 × 10 mm).

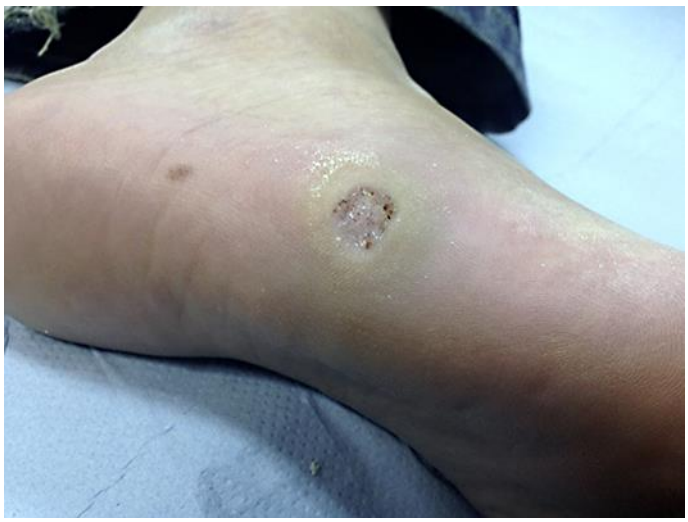


Fig. 2. Lesion at 3 weeks after treatment.

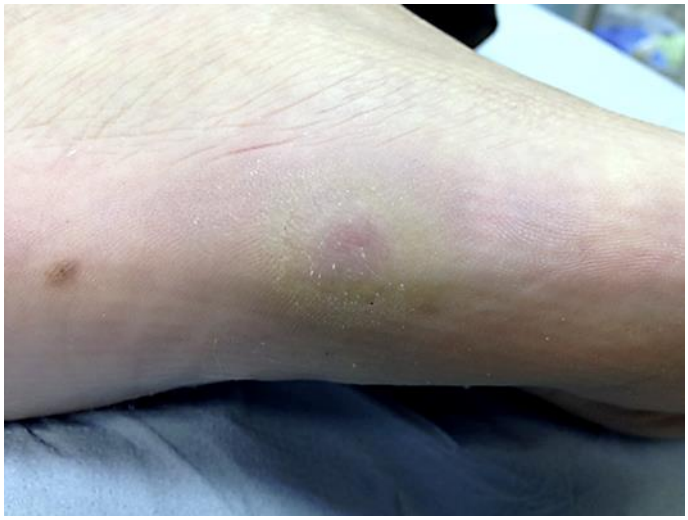


Fig. 3. Lesion at 5 weeks following 2 treatments.

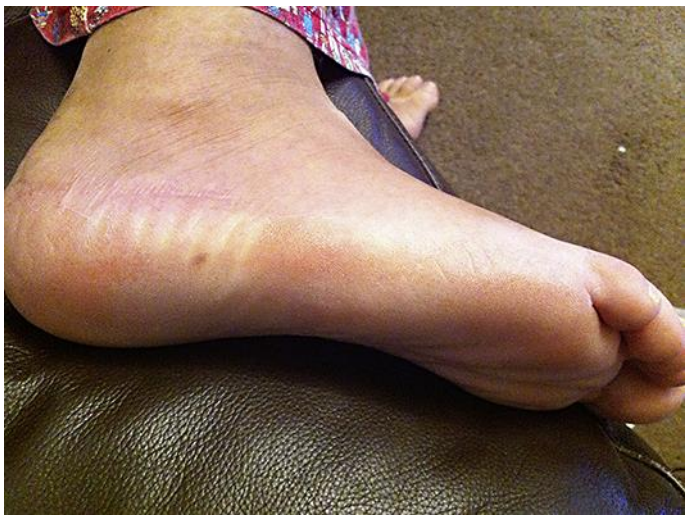


Fig. 4. Lesion resolved at second visit post-operatively.

Microwaves: A Painless, Efficient New Treatment for Plantar Warts

Independent research supports the effectiveness of this new technology.

BY ALEC HOCHSTEIN, DPM

Plantar warts (Verruca plantaris) are a familiar challenge for every podiatry practice. Patients turn up at the clinic in hopes of rapid relief from this painful, disfiguring condition, and are instead faced with numerous treatment options—all of which have uncertain success rates. Additionally, many of the common treatments are associated with pain and/or ongoing inconvenience. A new therapy created in Scotland offers an effective, noninvasive option, activating the power of the patient's own immune system to address the problem and clear the underlying virus. Below is an overview of the condition and available treatments, along with a detailed look at the new immune therapy.

Plantar warts can be particularly resistant to treatment, and up to one-third of them recur after being treated.

The Challenge of Plantar Warts

The human papilloma virus (HPV) is a persistent organism, and like most viruses, has no direct cure; the clinician can only treat the symptoms and wait for the patient's immune system to eliminate the underlying infection. Over 118 types of HPV have been identified, although only five strains typically cause warts on the feet and hands (Types 1, 2, 4, 27 and 57)[1]. No vaccines exist for these strains. Patient susceptibility to HPV is directly related to the strength of the immune system. People with compromised immune systems are more likely to develop warts than are healthy people, and children are more susceptible than are adults.

Plantar warts can be particularly resistant to treatment, and up to one-third of them recur after being treated.[2] Appearing on weight-bearing surfaces of the foot, they grow inward and often cause considerable pain. The virus is contained within the lesion, 2 to 3 mm below the skin surface. This growth habit allows plantar warts to escape detection by the immune system, and for this reason they can persist for years.

Difficulty in resolving a problem inevitably gives rise to many types of treatments, and this is particularly evident in the case of warts. Even if warts are successfully removed, residual virus can cause their recurrence. Below is the catalog of wart removal methodologies that have been available up until now:

Topical Keratolytics

Salicylic acid is usually the first-line approach to treating warts, sometimes applied in combination with urea, lactic acid, mono- and trichloroacetic acid, or cantharidin. Essential oils, vinegar, duct tape, fluorourasil, silver nitrate, zinc oxide[3], Vitamin A (retinoids) derived from fish oil[4] and numerous other remedies are also applied to warts. These treatments all require prolonged applications, multiple times each day, over a period of months. Sometimes they cause the supra dermal portion of the wart to be shed, but it often grows back.

Cryotherapy

This method freezes the wart and destroys the cells, typically using liquid nitrogen. Local anesthesia is sometimes needed for this procedure, which usually causes some damage in surrounding tissue.

Immunotherapy

A variety of immunotherapy agents are administered by injection, including the measles-mumps-rubella vaccine (MMR), raw Trichophyton antigen and/or Candida albicans antigen. Needling the wart (under local anesthesia) pushes the virus deeper into the body, in hopes of activating the person's immune system. A variety of topical immunomodulators such as imiquimod (Aldara) are also used off-label to treat plantar warts, with mixed results.[5]

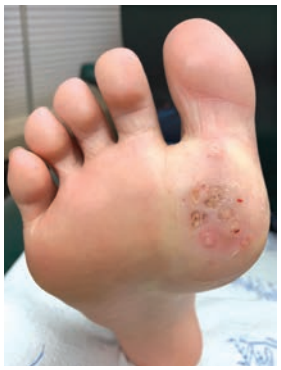
Continued on page 64

New Concepts and Studies

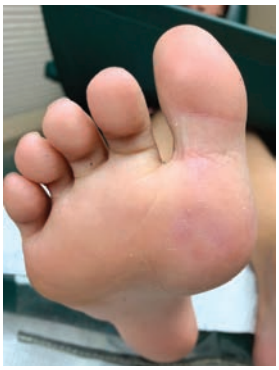
"New Concepts" is a forum for the presentation of (1) new technologies and products and (2) new studies involving existing products. Readers should be aware that Podiatry Management *does not specifically endorse any of the technologies, concepts, or products being discussed.*

Electrocautery and Lasers

Pulsed dye lasers and carbon dioxide lasers have been used to either excise or vaporize verruca lesions, but research[1] indicates that the overall results are not superior to keratolytic topical methods. Similarly, electrocautery is effective at removing the existing wart, but doesn't affect its recurrence, and is also somewhat traumatic to the patient.



15 year old female with multiple plantar verruca



Same patient 1 month after one application of Swift Emblation Treatment

Excision

In some cases, surgery is used for warts, although even this often turns out to be a temporary solution. In the meantime, the patient may not be able to bear weight on the surgical site during healing, and there is also the issue of potential infection.

New Option: Swift Portable Microwave Device

It's clear from the multiplicity of approaches reviewed above that no reliable solution to plantar warts has yet presented itself. As of January 2019, a new treatment modality called Swift has become available in the United States.

This relatively small (9 lb) device, made by Scotland's Emblation Ltd., provides a precise and effective treatment for warts. The clinician uses a 7 mm applicator to apply 8-10W (watts) of microwave energy directly to the affected skin area. The microwaves penetrate the tissues to a predetermined depth of 3.2mm, heating cells to between 109 to 114 degrees F. This is enough heat to cause proteins inside the virus-infected cells to be released, and the patient's immune system then becomes aware of the presence of the virus. Each application lasts only two seconds. A typical treatment session involves five of these 2-second applications, at intervals of one second. Patients typically experience complete remission after three to four such sessions spaced four weeks apart in order to align with the patient's immune cycle.

Microwaves Inside the Body?

Microwaves operate at a very low frequency (8 GHz) when compared to other types of electromagnetic radiation. Situated on the energy spectrum between radio waves and infra-red waves, microwaves cannot cause any damage to living DNA. When they enter the body from the Swift applicator, they agitate water molecules and cause some friction. They do not break the surface of the skin. The same type of radiation used by Swift is also being used around the world in cutting-edge soft tissue treatments for lung, liver, kidney and breast cancer.

Comparative Benefits of Swift

- Office visits are shortened, because the procedure is simple. No pre-treatment preparation beyond a light debridement is required, and the non-invasive procedure does not require any post-treatment dressing of any kind. While there is some pain during the treatment itself, there is no post-procedural pain experienced.
- Patients are fully mobile immediately following treatment. Research by the company's U.S. distributor indicates that 98 percent of patients continue all their daily activities at a normal level following Swift treatment.
- No smoke or burning odor, such as can occur after electrocautery or laser.
- No sterilization procedures are needed, because a new applicator tip is used for each patient.
- No breaks in the skin are created, so dressings are not required and infection is not a risk.

The Swift Microwave Therapy System

- Microwaves penetrate at a predetermined depth of 3.2mm, no risk of lateral spread/damage.
- Microwaves are better at denaturing the HPV virus than lasers.
- Mosaic warts can be treated in one session, rather than requiring multiple excisions.
- Swift is equally appropriate for all types of warts. It works. Clinical studies on recalcitrant plantar warts indicate a 75.9% clearance rate; however, real world clinical data suggest 85% efficacy.

Developed in Partnership with Clinicians

Swift was developed through six years of research in the UK, and has undergone thousands of hours of evaluation by podiatrists and dermatologists in that country. The Scottish government and private investors have offered support through this development process, and the result is a tool that genuinely transforms the way that warts are treated. Excellent results have been realized throughout the UK, Canada, and Australia—and as of January 2019, Swift is now available to providers in the United States. Saorsa, Inc, based in Seattle, partners with Emblation to offer the technology in this country. Saorsa has partnered with Western University and Kent State University of Podiatric Medicine as well as performed treatments at Temple University.

Continued on page 66

Supported by Independent Research

In one independent study, published in Case Reports in Dermatology[6], an adult patient had a painful plantar wart, 10 mm in diameter, on the right styloid process. This wart had been present for over a year, and had not responded to cryotherapy. Microwave energy (50 J [10 W for 5 s]) was applied by means of the Swift applicator, directly onto the surface of the wart. A 75 percent improvement in pain was experienced almost immediately. Three weeks later, a second treatment was given. Pain had completely disappeared by two weeks following that second treatment, and at a six-month follow-up there was no further evidence of the wart.

For clinicians, it is professionally rewarding to be able to offer an effective, efficient treatment for a condition that many patients struggle with for years. Such a revolutionary modality brings financial rewards as well; Saorsa provides an ROI calculator on their website, providing complete transparency with respect to the balance between the device's cost (to purchase or lease) and the increased income that results. The treatment is new and is non-destructive and therefore not covered by any insurer at this point. This eliminates the time demands of insurance paperwork.

With today's advances in biotechnology, it is time to move past the "armamentarium"[1] of partially-effective treatments for verrucae. A true solution to viral infection can arise only from engaging the body's own immune response, and Swift offers the best tool to activate that engagement. **PM**

Footnotes

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Disclosure: Dr. Hochstein is a key opinion leader and consultant for Saorsa—North American Distributor of Swift Technology.

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Article accepted on 17/03/2017

Microwave therapy for cutaneous human papilloma virus infection

Background: Human papilloma virus (HPV) infects keratinocytes of the skin and mucous membranes, and is associated with the induction of cutaneous warts and malignancy. Warts can induce significant morbidity and disability but most therapies, including cryotherapy, laser, and radiofrequency devices show low efficacy and induce discomfort through tissue destruction. Microwaves are readily capable of passing through highly keratinised skin to deliver energy and induce heating of the tissue in a highly controllable, uniform manner. *Objectives*: To determine the effects of microwave on cutaneous HPV infection. *Materials & methods*: We undertook a pilot study of microwave therapy to the skin in 32 consecutive individuals with 52 recalcitrant long-lived viral cutaneous warts. Additionally, we undertook a molecular characterisation of the effects of microwaves on the skin. *Results*: Tissue inflammation was minimal, but 75.9% of lesions cleared which compares favourably with previous studies showing a clearance rate of 23-33% for cryotherapy or salicylic acid. We show that microwaves specifically induce dendritic cell cross-presentation of HPV antigen to CD8+ T cells and suggest that IL-6 may be important for DC IRF1 and IRF4 modulation to enhance this process. *Conclusion:* Keratinocyte-skin dendritic cell cross-talk is integral to host defence against HPV infections, and this pilot study supports the concept of microwave induction of anti-HPV immunity which offers a promising approach for treatment of HPV-induced viral warts and potentially HPV-related cancers.

Key words: warts, microwave, CD8+ T cells, HPV

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Cutaneous HPV infection is common and warts are thought to affect most people at some time during their lives. Point prevalence estimates range from 0.8% to 4.7% of the population and two million people seek medical advice about warts each year in the UK [1], yet treatment options are poor and a meta-analysis has shown no significant benefit over placebo [2]. Although skin is most frequently infected by "non-oncogenic" HPV, most HPV-associated skin squamous cell carcinomas are diagnosed in persistent and recalcitrant verrucae and the majority contain HPV16 [3].

HPV infects the basal epithelial cells of cutaneous and mucosal keratinised epithelia and infection is mainly controlled by T cell-mediated immunity [4]. HPV-specific CD8+ lymphocytes are critical for clearance of HPV viral warts [4] and individuals treated with immunosuppression to prevent organ graft rejection do not clear HPV infections. In healthy individuals, induction of HPV-specific CD8+ T cells with topical imiquimod (TLR7 agonist) has been shown to facilitate wart clearance [5, 6]. However, tissue penetration is a limiting factor for the therapeutic potential of imiquimod on most non-mucosal sites.

Other modalities of thermal ablation have previously been investigated for the treatment of warts [7-10]. Direct heat ablation is now rarely used because of scarring and subsequent morbidity. The most widely used physical modality is liquid nitrogen application (cryotherapy) to the skin [11]. This causes tissue destruction and in a recent meta-analysis of randomised controlled trials, this therapy has been shown to have low efficacy in the management of common warts (with a mean clearance on all sites of 49%) [12]. Microwaves (30 MHz to 30 GHz) exist in the electromagnetic spectrum between radiofrequency and visible light and have been widely used as a means for delivering heat energy to induce thermal ablation in the treatment of cancer, especially for inoperable liver tumours [13], but have not been previously applied to skin. Recent technological advances have enabled development of a hand-held device to deliver targeted application of microwave therapy to skin. We set out to test the potential of this new modality as a treatment for warts in a Phase 1, open-label, uncontrolled clinical study. It was observed in the first few cases that the warts shrank and resolved without obvious necrosis, tissue damage, or inflammation. Hence, we hypothesised that somehow anti-HPV immunity was being activated. We therefore undertook morphological and histological analysis of microwave-treated human skin and investigated for evidence of enhanced anti-HPV immunity. We demonstrated that, even at low energy levels, microwave therapy potentiates cutaneous immunity to HPV.

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Methods and materials

Patients and *in vivo* microwave treatment

The study was approved by the local research ethics committee in accordance with the declaration of Helsinki. Individuals with treatment-refractory plantar warts were recruited. The diagnosis of plantar wart was confirmed by a podiatrist experienced in management of such lesions. A clinically significant wart was defined as >one year duration, with at least two previous failed treatments (salicylic acid, laser, cryotherapy, needling, and surgical excision). Exclusions were pregnancy or breast feeding, pacemaker *in situ*, metal implants within the foot or ankle, co-morbidities affecting immune function, or capacity to heal. At each study visit, a complete examination of the affected area was undertaken and a quantitative measure of pain and neuromuscular function assessed. No dressing was required and volunteers continued normal everyday activities after treatment with no restrictions.

A total of 32 volunteers with 54 foot warts were enrolled into the study (17 males and 15 females; age range: 22-71 years; mean: 44·79 years [SD: 13.019]). Sixteen were solitary and 38 multiple-type warts (*e.g.* mosaic verrucae). Mean lesion duration was 60.54 months (range: 12-252) and diameter 7·43 mm (range: 2-38 mm; SD: 6.021). At the conclusion of the study period, one patient had been lost to follow-up and two patients had withdrawn (n = 3; four warts) but were retained in the statistical analysis, classified as unresolved lesions.

Microwave treatment (Swift®, Emblation Medical Ltd., UK) of the most prominent plantar wart was titrated up, as tolerated to 50 J over a 7-mm diameter application area (130 J/cm^2) over 5 seconds (10 watts for 5 seconds). Lesions >7 mm received multiple applications until the entire surface of the wart had been treated. If the wart persisted, treatment was repeated at one week, one month, three months, and 12 months. Response to treatment was assessed by the same investigator as binary; "resolved" or "unresolved". Resolution was indicated by fulfilling three criteria: (1) lesion no longer visible; (2) return of dermatoglyphics to the affected area; and (3) no pain on lateral compression. Pain was assessed using a 10-point visual analogue scale.

Human skin and blood samples

Skin and blood samples for microwave experiments were acquired from healthy individuals as approved by the local Research Ethics Committee in adherence to Helsinki Guidelines.

Histological analysis

Skin samples were treated immediately *ex-vivo* with microwaves (Swift s800; Emblation Ltd., UK) or liquid nitrogen therapy and punch biopsies taken from treated skin were sent for histological analysis or placed in culture media.

Histological analysis of hematoxylin and eosin (H&E)-stained tissue sections was undertaken following fixation and embedding in paraffin wax. DNA damage was assessed by staining for single-stranded and double-stranded DNA breaks by TUNEL assay using the ApopTag® In Situ Apoptosis Detection Kit (Millipore, UK). Following culture, supernatants were collected and analysed for lactate dehydrogenase (LDH) release using the Cytotoxicity Detection Kit (Roche applied science) as a measure of apoptosis.

Cell culture and *in vitro* microwave treatment

Primary keratinocytes were obtained from pooled neonatal foreskin donors (Lonza, Switzerland) and cultured in keratinocyte growth medium 2 (PromoCell) at 37°C, 5% CO_2, until 70-90% confluency for use in experimental work (P4-P10).

Human skin explant cultures and human HaCaT keratinocytes were cultured in calcium-free DMEM (ThermoFisher Scientific) with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 10% foetal bovine serum (FBS), and supplemented with calcium chloride at 70 μM final concentration.

Microwave treatment of cells in culture was delivered in a flat-bottomed well using the Swift device applied directly to the plastic base from the underside. To assess whether the plastic caused loss of microwave energy in our system, the 150 J Swift programme applied through the culture well base delivered a temperature rise of 18.6°C (SD: 1.1) to 200 g of culture media, equivalent to ∼15.61 J (SD: 0.92). Thus, it could be estimated that 15 J applied *ex vivo* would be equivalent to ∼150 J as tested here *in vivo*. However, energy loss during skin application would reduce this difference, but calculation of the precise transfer of energy to skin *in vivo* was not possible, so we estimate that the dose delivered *in vitro* is up to 10-fold lower than that by direct skin application *ex vivo*. To avoid confusion, the setting on the Swift system is the energy level referred to throughout the manuscript (in human and *in vitro* studies).

Lymphocytes were cultured in RPMI-1640 medium with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 292 μg/mL L-glutamine, supplemented with 10% FBS or 10% heat-inactivated human serum (HS). HaCaT cells were cultured to sub-confluency to avoid cell differentiation and used in assays at passage 60-70. Cells were plated at 2.5×10^3 cells/well in 96-well flat plates (Corning Costar) and cultured overnight to reach confluence. HaCaTs were washed once with PBS before treatment with microwaves, liquid nitrogen (10 seconds), or with LPS+IFN-γ (1 ng/mL+1,000 U/mL). Cells were cultured for 24 hours before supernatants were harvested. HPV16 E7 protein was expressed in *E. coli* at the Protein Core Facility of Cancer Sciences Unit, University of Southampton. Endotoxin was removed using Detoxi-Gel endotoxin removal using columns (Thermo Scientific).

For HPV-specific T cell lines, PBMCs were isolated from HLA-A2-positive individuals, as previously described [14]. PBMCs were seeded at $2\text{-}4 \times 10^6$ cells/well in 24-well culture plates and 10 μg/mL ninemer HLA-A2-restricted HPV16 epitope LLM (LLMGTLGIV) [15] was added; cells were cultured in 1 mL RPMI+10% HS. On Day 3, cells were fed with RPMI+10% HS+IL-2 (200 IU/mL), and then fed again on Day 7 or when needed. After Day 10, HPV-specific T cells were harvested for cryopreservation before testing against HPV using ELISpot assays.

To generate monocyte-derived dendritic cells (moDCs), CD14+ cells, were positively isolated from PBMCs by

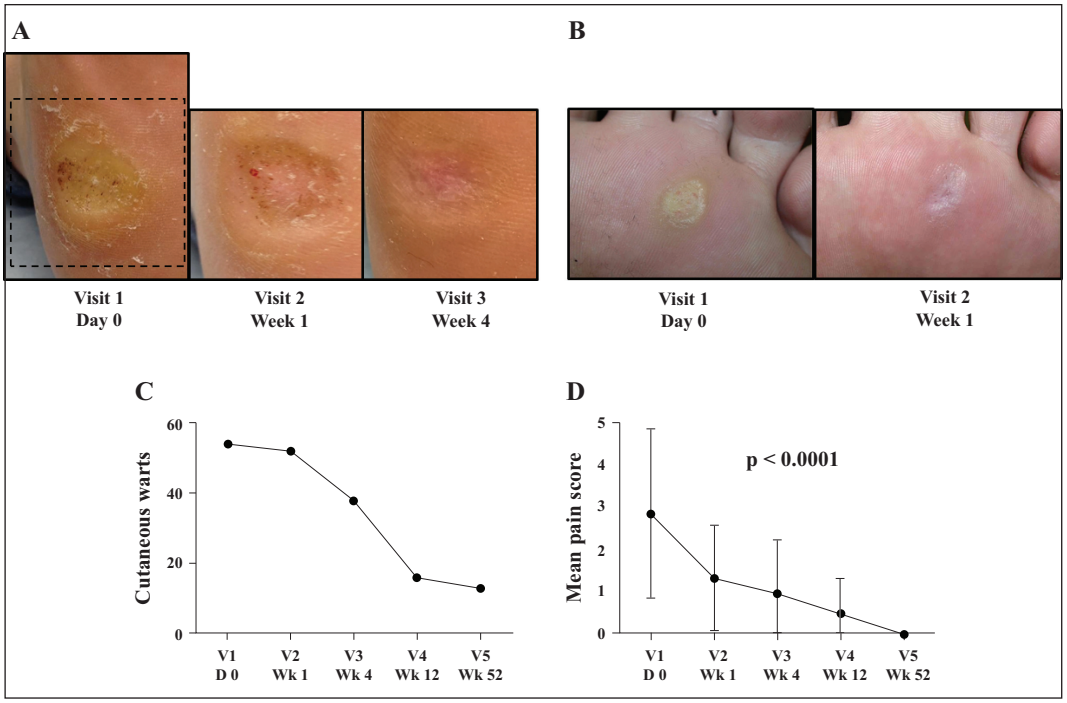

Figure 1. Response of recalcitrant warts to microwave therapy. **A)** Clinical image of plantar wart pre-microwave treatment (left), after one treatment (middle), and after two treatments (right). **B)** Clinical image of plantar wart pre-microwave treatment (left) and after one treatment (right). **C)** Intention to treat analysis of 32 patients with 54 HPV foot warts treated by microwave therapy over five visits: baseline, one week, one month, three months, and 12 months. Resolved warts were enumerated. **D)** Pain scores were assessed using a 10-point visual analogue score at each visit. Statistical test: one-way ANOVA.

magnetic separation using CD14 microbeads (Milentyi Biotec, UK), according to the manufacturer's protocol. Cells were washed and resuspended in RPMI+10% FBS+250 U/mL IL-4 and 500 U/mL GM-CSF. At Day 3, cells were fed with RPMI+10% FBS+IL-4 and GM-CSF, and then harvested on Day 5 for use in functional assays.

ELISpot, flow cytometry and qPCR

Keratinocytes (HaCaTs or primary as indicated) were treated with microwaves at various energy settings before removal of supernatant at various time points. MoDCs were treated overnight with keratinocyte supernatant, then washed twice before incubation with LLM peptide (10 μg/mL for 2 hours) or HPVE7 protein (10 μg/mL for 4 hours) before a further wash. Human IFN-γ ELISpot (Mabtech, Sweden) was undertaken, as per the manufacturer's protocol and as reported previously [14]. moDCs at 1×10^3 were plated with autologous HPV peptide-specific T cells at a ratio of 1:25. Spot forming units (sfu) were enumerated with ELISpot 3.5 reader (AID, Germany).
MoDCs were treated with HaCaT supernatant and harvested at 24 hours for flow cytometric analysis of cell phenotype. Cells were stained with violet LIVE/DEAD stain (Invitrogen, ThermoFisher, UK) for 30 minutes at 4°C, then washed with PBS+1% BSA and stained with antibodies PerCP-Cy5.5 anti-HLA-DR, FITC anti-CD80, FITC anti-CD86, or PE anti-CD40 (Becton Dickinson, UK) for 45 minutes at 4°C. Cells were washed, then resuspended in PBS+1% BSA, and analysed using the BD FACSAria and the FlowJo v10.0.08 analysis software.

The expression of chosen genes was validated with quantitative PCR using the TaqMan gene expression assays for target genes: *YWHAZ* (HS03044281_g1), *IRF1* (Hs00971960_m1), and *IRF4* (Hs01056533_m1) (Applied Biosystems, Life Technologies, UK) in human skin, and treated as indicated. RNA extraction (RNeasy mini kit, Qiagen) and reverse transcription (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems; ThermoFisher Scientific UK)) were carried out accordingly to the manufacturer's protocol.

Results

Treatment of human papilloma virus infection in humans with microwave therapy

Of the 32 volunteers with severe warts, 54 treatment-refractory plantar warts were treated with microwave therapy (*figure 1A, B*). At the end of the study period, of the 54 warts treated, 41 had resolved (75.9%) and nine remained unresolved (16.7%), and two patients (with three warts [5.6%]) withdrew from the study and one patient (with one wart [1.9%]) was lost to follow-up. The mean number of days to resolution was 79.49 days (SD: 34.561; 15-151 days). Of the resolving lesions, 94% had cleared after three treatments (*figure 1C*). No significant difference in resolution rates between males and females (p = 0·693) was observed. Statistically significant reductions in pain were observed as treatment progressed (p<0.0001) (*figure 1D*). Adverse events were minimal. One patient reported

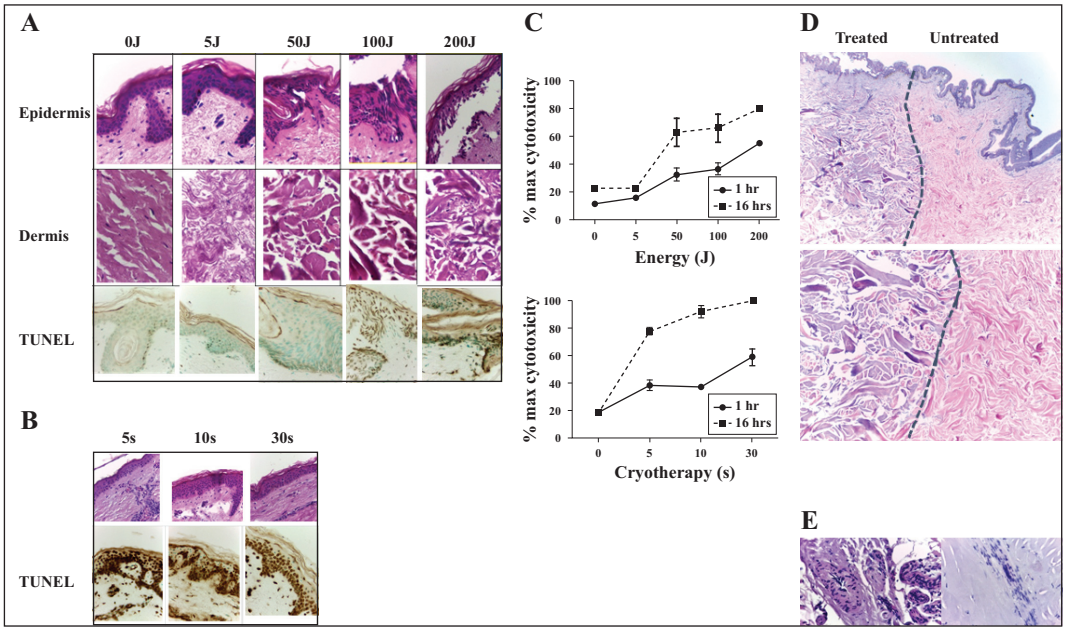

Figure 2. Microwave effects on human skin. **A)** Histological analysis of normal human skin treated with microwave stimulation visualised in the epidermis/papillary dermis (upper and lower panels), or deep dermis (middle panels). Skin was subjected to microwave therapy (0-200 J) before punch excision. Tissue was cultured for one hour before fixation and paraffin embedding (H&E or TUNEL staining; original magnification: ×20). **B)** Histological analysis of human skin treated with liquid nitrogen therapy for 5, 10, or 30 seconds, before punch excision. Tissue was cultured for one hour before fixation and paraffin embedding (H&E or TUNEL staining; original magnification: ×20). **C)** Following microwave therapy (upper panel) or cryotherapy (lower panel), skin samples (in triplicate) were excised and cultured in media for one or 16 hours before measurement of cytotoxicity, assessed by harvesting supernatant to measure supernatant lactate dehydrogenase (LDH) release by ELISA. **D)** Skin was subjected to microwave therapy (150 J) before punch excision at the margin of the treated zone. Tissue was cultured for one hour before fixation and paraffin embedding (H&E stain; original magnification: ×10 [upper panel], x100 [lower panel]. **E)** Skin was subjected to microwave therapy (150 J) before punch excision. Tissue was cultured for one hour before fixation and paraffin embedding (H&E stain showing deep dermis adnexae: glandular [left] and vascular [right]; original magnification: ×100). Data are representative of three independent experiments.

transient pain from the treatment which required a simple oral analgesic (paracetamol) and resolved within 24 hours. This individual withdrew from the study. No further adverse events were reported. No cases of scarring were recorded following completion of treatment. No cases of neuromuscular dysfunction were reported.

Microwave treatment of human skin

Human skin has not previously been reported to be treated with microwave therapy, therefore, we proceeded to undertake a full histological analysis of treated skin. Skin removed during routine surgery was treated *ex vivo* and one hour after treatment punch biopsies were taken and fixed for histological processing. Neither macroscopic nor histological changes were noted with the lowest energy setting (5 J). At 50 J, mild macroscopic epidermal changes only were noted, and microscopically minor architectural changes and slight elongation of keratinocytes were seen without evidence of altered dermal collagen. At higher energies (100/200 J), gross tissue contraction was visible macroscopically. Microscopic changes in the epidermis were prominent, showing spindled keratinocytes with linear nuclear architectural changes and subepidermal clefting (*figure 2A*). Dermal changes were prominent at energies of 100 J and above and showed a homogenous hyalinised zone of papillary dermal collagen, thickened collagenous substances, and accentuation of basophilic tinctorial staining of the dermal collagen with necrotic features (*figure 2A*). These features are similar to electro-cautery artefacts and suggest that at >100 J, there is the potential to coagulate proteins and induce scarring. Histological analysis both at 16 hours and 45 hours showed similar changes (data not shown).

In clinical practice, cryotherapy is delivered to the skin by cryospray, which is time-regulated by the operator. In contrast to microwave therapy, minimal epidermal or dermal architectural change was identified with cryotherapy at standard treatment duration times (5-30 seconds), but did show a dose-dependent clumping of red blood cells in vessels (*figure 2B*).

Tissue release of LDH acts as a biomarker for cellular cytotoxicity and cytolysis. To examine the extent of cell death induced by microwave irradiation, human skin was treated with 0, 50, 100 or 200 J before punch excision of the treated area and incubation in medium for one hour or 16 hours. Measurement of LDH revealed a dose-dependent induction of tissue cytotoxicity with increasing microwave energies (*figure 2C*). In line with the lack of histological evidence of cellular damage, at 5 J, cytotoxicity of microwave application was equivalent to control. Early cytotoxicity was not prominent at 50 J, but became more evident after 16 hours. Higher energy levels induced more prominent cytotoxic damage. In contrast to microwave therapy, liquid nitrogen

treatment of skin induced cytotoxicity at the lowest dose both at one hour and 16 hours.
Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) identifies cells in the late stage of apoptosis. Analysis at 0, 5, 50, 100 and 200 J identified increased cellular apoptosis in the epidermis above 100 J (*figure 2A*). In contrast, cryotherapy induced significant epidermal and dermal DNA fragmentation (*figure 2B*).
The physics of microwave therapy suggests a sharp boundary between treated and untreated tissue with minimal spreading of the treated field. This was borne out histologically by a clear demarcation between treated areas extending vertically from the epidermis through the dermis (*figure 2D*). Examination of the dermis showed that microwave therapy modified skin adnexae, inducing linear nuclear architectural changes in glandular apparatus, micro-thrombi, fragmented fibroblasts, and endothelial cells (*figure 2E*).

Microwave induction of immune responses in skin

We first examined the response of keratinocytes to microwave therapy *in vitro*. In analysing *in vitro* the effects of microwave therapy, it was necessary to apply the microwave treatment through culture dish plastic. Thus, the energy setting *in vitro* is equivalent to a lower energy setting than with direct application *in vivo* (*see above*). In keratinocyte monolayers (HaCaT), apoptosis was induced by microwave therapies above 100 J *in vitro* (*figure 3A*). Only above the apoptotic threshold (100 J) were surface phenotypic changes of cellular activation noted in viable cells with increased expression of HLA-DR, CD40, and CD80 (*figure 3B*). Next, we utilised a model of skin cross-talk of keratinocyte signalling to dermal dendritic cells. Initially, we observed strong activation of MoDCs primed with supernatant from microwave-treated keratinocytes (*data not shown*), but we wished to disentangle the pro-inflammatory effects of apoptosing/necrotic cells from viable cell cross-talk. Therefore, keratinocytes were treated with microwave therapy as above, and washed after eight hours to remove dead or apoptotic cells. Treated keratinocytes were then incubated for a further 16 hours before supernatant collection to prime moDCs, which had not been directly exposed to microwave therapy. The supernatants induced potent induction of moDC activation with increased expression of CD86, CD80, and to a lesser extent, CD40 (*figure 3C*).
We next set out to model the functional outcome on skin dendritic cells following microwave treatment of keratinocytes. Keratinocyte monolayers (HaCaT) were untreated, or microwave- or cryotherapy treated before supernatant harvesting. Supernatant-primed DCs were pulsed with a nine-amino acid HLA-A2 epitope (LLM) from human papilloma virus (HPV) E7 protein and cultured with an autologous HPV-specific CD8+ T cell line. As expected, in all conditions, the moDCs efficiently presented HPV peptide to HPV-specific CD8+ T cells, inducing IFNγ (*figure 4A*). However, dendritic cell presentation of HPV is dependent upon cross-presentation to the MHC class I pathway. Therefore, we also tested the capability of untreated, microwave-treated or cryotherapy-treated KC-primed moDCs to present HPV E7 protein to an HLA-matched HPV-specific CD8+ T cell line. Strikingly, only microwave-treated KCs were capable of priming moDCs to enhance cross-presentation (*figure 4B*). To explore the potential mechanism of keratinocyte response to microwave therapy, we confirmed up-regulation of HSP-70 in response to microwave therapy of keratinocytes (*figure 4C*). Although, the assay used did not distinguish constitutive from inducible HSP-70, we clearly demonstrated global increase in HSP-70 expression following microwave therapy. Additionally, IL-6, but not IL-1β or TNF-α, was expressed in response to microwave stimulation, which suggests that alternative inflammatory signalling pathways from that seen in cryotherapy-treated cells are induced by microwave stimulation (*figure 4D*). To further explore the potential innate immune signalling pathways in keratinocytes following microwave therapy, we examined IRF1 and IRF4. These transcription factors are key regulators of dendritic cell activation of adaptive immunity. We show that microwave therapy induced down-regulation of IRF1 and up-regulation of IRF4 (*figure 4E*).

Discussion

This is the first study to investigate the potential efficacy of locally delivered microwaves in the treatment of cutaneous viral warts. In this uncontrolled pilot study, we report a complete resolution rate of 75.9% of recalcitrant plantar warts (with an average lesion duration of over five years). This compares very well with previous reports of plantar wart resolution for salicylic acid and or cryotherapy (23-33%) [16].
For all novel therapies, adverse events are critical but we did not identify a strong signal for adverse events. As with current physical treatments for warts, discomfort is expected for the patient. During the study, patients generally reported that for a typical five-second treatment, they endured moderate discomfort for approximately two seconds, which immediately diminished after the treatment had completed. In addition, it was commonly noted that discomfort was less with subsequent treatments. One male patient withdrew from the study after one treatment, citing the pain of treatment as the reason. In the study design phase, pre-operative use of topical anaesthetic cream was tested, but appeared to do little to mitigate the pain (*unpublished data*) and it was felt that the pain of local anaesthetic injection would exceed that normally experienced during a microwave treatment. Following microwave therapy, patients did not require dressings or special advice as no wound or ulcer was caused, allowing the patient to continue normal activity. The short microwave treatment time (five seconds) offers a significant clinical advantage over current wart therapies, such as cryotherapy and electro-surgery. Within five seconds, microwaves penetrate to a depth of over 3.5 mm at the energy levels adopted for the study [17]; possibly a greater depth than can be attained by cryosurgery or laser energy devices. Moreover, microwaves, like all forms of electro-magnetic radiation, travel in straight lines and energy is deposited in alignment with the "beam" emitted from the device tip with little lateral spread, meaning minimal damage to surrounding tissue, as confirmed in this study. Microwaves induce dielectric heating. When water, a polar molecule, is exposed to microwave energy, the

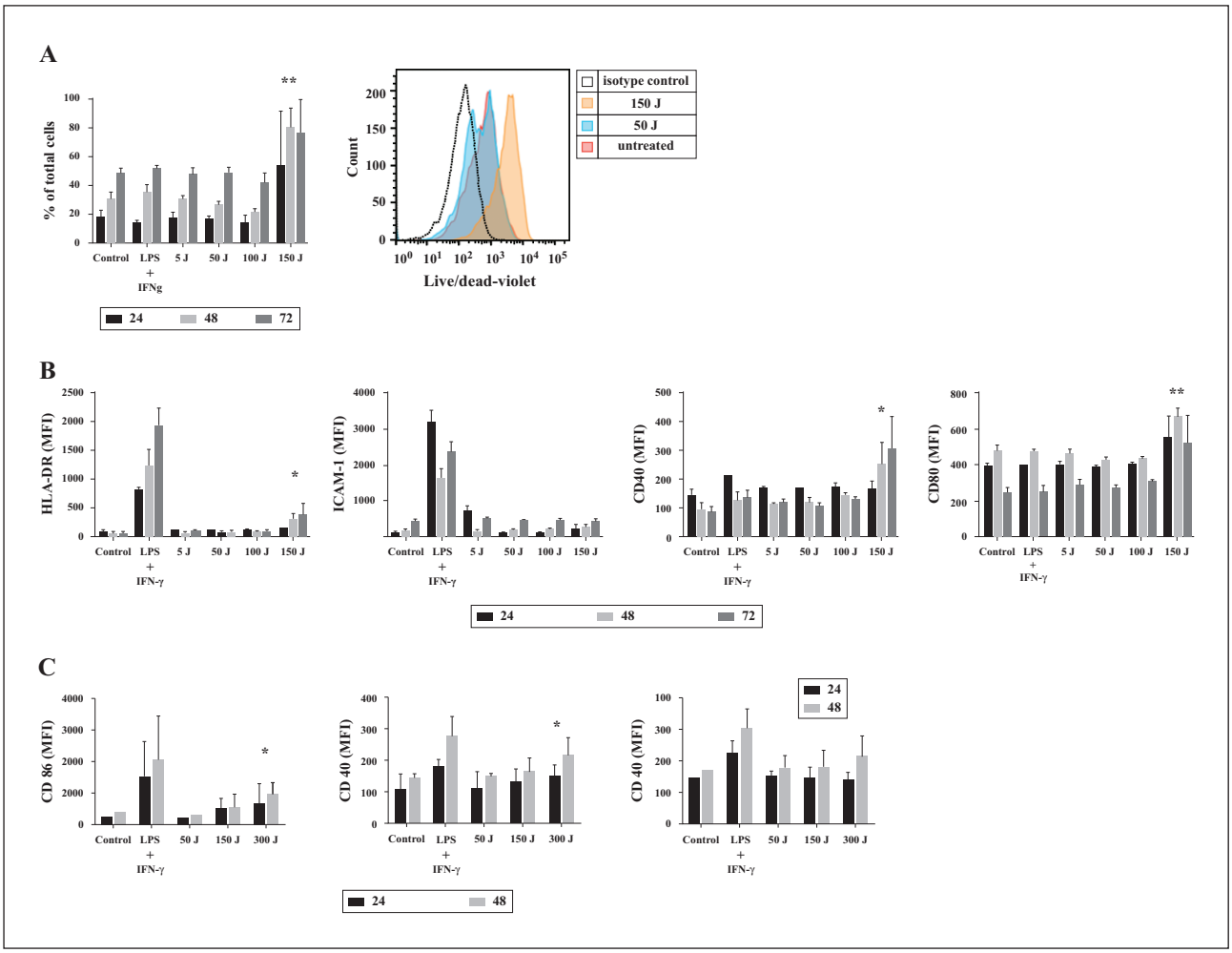

Figure 3. Microwave activation of keratinocytes and dendritic cells. **A)** Left: flow cytometric analysis of viable keratinocytes (% of total cells) indicated by negative staining with the amine reactive viability dye LIVE/DEAD after control, microwave (5-150 J), or LPS/IFN-γ treatment. Keratinocytes were treated then kept in culture for 24 (black bars), 48 (light grey) or 72 (dark grey) hours before analysis. Right: flow cytometric analysis of keratinocyte viability after microwave therapy or control, depicted as a histogram. X-axis: LIVE/DEAD stain; y-axis: cell count. **B)** Flow cytometric analysis of HLA-DR, ICAM-1, CD40 or CD80 expression on viable keratinocytes. Keratinocytes were treated with microwave therapy (5-150 J), LPS/IFN-γ, or nil (control), rested in culture for 24 (black bars), 48 (light grey) or 72 (dark grey) hours, before analysis of the viable population. **C)** Flow cytometric analysis of CD86, CD80, and CD40 expression on viable monocyte-derived dendritic cells (moDCs). Keratinocytes were treated with microwaves (5-150 J), LPS/IFN-γ, or untreated (control), rested in culture for eight hours, then washed. They were left in culture for the remaining time until 24 (black bars) or 48 (light grey) hours, before transfer of supernatant onto moDCs. MoDCs were incubated for 24 hours before harvesting for analysis. Data are representative of three independent experiments. Mean+SD; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

molecule is excited and rotates to align with the alternating electro-magnetic field. At microwave frequencies, the molecule is unable to align fully with the continuously shifting field resulting in heat generation. Within tissues, this acts to rapidly elevate temperatures. This process increases cellular temperature because it does not depend on tissue conduction. Microwave treatment produces no vapour or smoke unlike ablative lasers and electro-surgery, eliminating the need for air extraction systems due to the risk of spreading viral particles within the plume [18].

Although microwave therapy has been considered a tissue ablation tool, we observed minimal skin damage after treatment with 50 J, yet good clinical responses were seen. Therefore, we investigated whether there was evidence to support an induction of anti-HPV immunity by microwave therapy. The critical nature of CD8+ T cell immunity for host defence against HPV skin infection is well established and supported by the observation of increased prevalence of infection in immunosuppressed organ-transplant recipients [19], and that induction of protection from HPV vaccines is mediated by CD8+ T cells [20]. We show here that microwave therapy of skin induces keratinocyte activation and cell death through apoptosis. However, *in vitro* microwave-primed keratinocytes are capable of signalling to dendritic cells and enhancing cross-presentation of HPV antigens to CD8+ lymphocytes at microwave energy levels equivalent to or lower than that used in the clinical study, which offers a potential explanation for the observed response rate in our clinical study. *In vitro* evidence suggests that this is likely

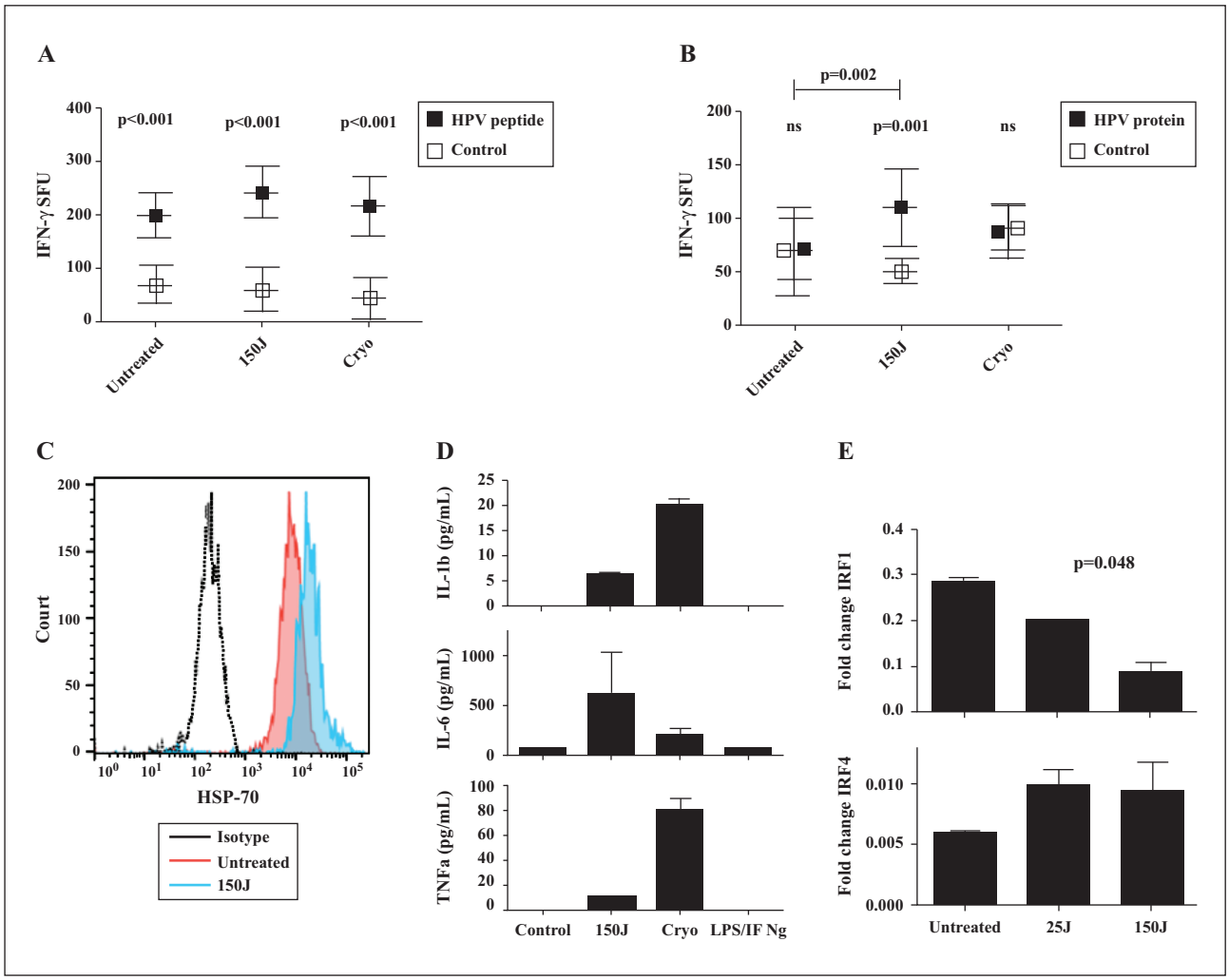

Figure 4. Microwave induction of HPV antigen cross-presentation. **A)** ELISpot assay of IFN-γ production by HPV-specific CD8+ cells following co-culture with HPV peptide-pulsed moDCs primed by supernatant from untreated, microwave-treated (150 J) or cryotherapy (cryo)-treated HaCaT keratinocytes. MoDCs were primed with supernatant for 24 hours prior to pulsing with control (open squares) or HPV peptide (closed squares) for two hours. Pulsed moDCs were then co-cultured with an HLA-matched CD8+ HPV-specific T cell line before assay with IFN-γ ELISpot. Statistical significance was determined using the Holm-Sidak method, with alpha = 5%. Data are representative of three independent experiments (mean+SD). **B)** ELISpot assay of IFN-γ production by HPV-specific CD8+ cells following co-culture with HPV E16 protein-pulsed moDCs primed by supernatant from untreated, microwave-treated (150 J) or cryotherapy (cryo)-treated HaCaT keratinocytes. MoDCs were primed with supernatant for 24 hours prior to pulsing with control (open squares) or HPV protein (closed squares) for two hours. Pulsed moDCs were then co-cultured with an HLA-matched CD8+ HPV-specific T cell line before assay with IFN-γ ELISpot. Statistical significance was determined using the Holm-Sidak method, with alpha = 5%. Data are representative of three independent experiments (mean+SD). **C)** Flow cytometric analysis of intracellular HSP-70 expression on viable keratinocytes after microwave therapy or control, depicted as a histogram. Primary human keratinocytes were treated with microwave therapy (150 J), or nil (untreated), rested in culture for 24 hours, before analysis. X-axis: anti-HSP-70; y-axis: cell count. **D)** ELISA of IL-6, TNFα, and IL-1β production by primary human keratinocytes 24 hours after treatment with microwave therapy (150 J), LPS/IFNg, cryotherapy or control (untreated). **E)** Fold expression of change of IRF1 and IRF4 over a housekeeping gene in normal human skin with microwave therapy (25 J and 150 J) by qPCR.

to be mediated by cross-talk between microwave-treated skin keratinocytes and dendritic cells, through induction of danger-associated molecular patterns (DAMPs), such as HSP-70 in keratinocytes, resulting in up-regulation of DC CD40 and CD80/86 and subsequent enhanced cross-presentation of HPV proteins to CD8+ T cells. Microwave therapy also specifically induced enhanced IL-6 synthesis from keratinocytes. IL-6, is a pro-inflammatory mediator, important in anti-viral immunity, which has been recently shown to induce rapid effector function in CD8+ cells [21]. Thus, IL-6 up-regulation may provide an important additional mechanism for microwave-induced anti-viral immunity. The intriguing contrast between cryotherapy and microwave therapy revealed a far greater release of IL-1β and TNF-α with cryotherapy which, in addition to the lesser IL-6 induction, may offer potential to utilise the treatments for different situations where IL-1β/TNF-α-driven inflammation may be preferable, or vice versa.

Additionally, the specificity of inflammatory pathways induced by each modality may explain why cryotherapy and microwave stimulation may not show equal effectiveness in the same disease.

IRFs have been shown to be central to the regulation of immune responses [22-24]. IRF4 is essential for differentiation of cytotoxic CD8+ T cells [25, 26], but up-regulation in dendritic cells has also been shown to enhance CD4+ differentiation [23], therefore, this pathway may potentially enhance both CD8+ immunity and T-cell help following microwave treatment. IRF1 expression has been previously reported to be modulated by HPV infection, but different models have shown opposite outcomes [27, 28]. We show down-regulation of IRF1 in human skin in association with a microwave therapy, which supports the proposal of IRF-1 as a therapeutic target in HPV infection [28].

This study is the first of its kind to study microwaves in the treatment of plantar warts *in vivo*. Further work to examine the immune infiltrate in microwave-treated warts is planned. Whilst we acknowledge the limitations of the uncontrolled, non-randomised design, the promising results shown here suggest that a randomised controlled study with a larger sample size is warranted to confirm the efficacy of this treatment. ■

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TREATING VERRUCAE EFFECTIVELY WITH MICROWAVE ENERGY ARE WE GETTING WARMER?

‘Those who cannot be cured by medicine can be cured by surgery. Those who cannot be cured by surgery can be cured by heat. Those who cannot be cured by heat are to be considered incurable’

Hippocrates

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The treatment of cutaneous warts has not significantly changed in decades. In 2000, Dyall-Smith [1] remarked how little verruca treatments had changed since the fifties and this still holds true today, perhaps with the additions of some newer topical antiviral drugs [2] and photodynamic therapies [3]. Over the years, podiatric training for the treatment of warts is still largely based on chemical means including salicylic acid, monochloracetic acid, trichloracetic acid and liquid nitrogen as cryotherapy.

Looking at the evidence for these modalities, years on, it does not make for particularly good reading. The latest guidelines published by the British Association of Dermatologists [4] in 2014 continue to review the common remedies such as salicylic acid and liquid nitrogen closely. Although success is reported as being ‘modest’, in most cases it offers a disappointing outlook for sufferers. Particularly notable is the lower response rates from plantar warts. Salicylic acid treatment has better outcomes than placebo, and response rates for both cryotherapy and salicylic acid are just over 30%. Moreover, the science explaining how they may work is lacking.

Cryotherapy, like chemical therapies, is something that has been taught for many years in podiatry as an established treatment for plantar warts. The latest review by Sterling [4] suggests how its effects are, at best, limited. Moreover, the likelihood of prolonged pain and blistering is always a possibility particularly when longer freeze times have been applied. It is this unpredictability that perhaps has resulted in its decline in podiatric practice. Cryotherapy, like the rest, is a reasonable treatment for warts but unfortunately not on the sole of the foot. Keratin is an excellent insulator, reducing the penetration of the cold temperatures and thereby protecting the underlying skin and virus from frost damage.

In 2015, the authors undertook a study using microwave as a treatment for plantar warts. This was an emerging technology that had been developed over several years. The use of this device switched from the cold treatment of liquid nitrogen to that of localised tissue heating utilising dielectric energy. Microwaves when applied to human tissue have the ability to agitate water molecules. As a polar molecule, water attempts to align with the electrical field, but because the oscillating frequency is in constant movement the water molecule is unable to rest. This effect causes the molecule to rotate rapidly and generate heat at a molecular level within the tissues.

The work from this clinical study of the device, conducted through the University of Southampton, demonstrated that this technique is capable of eradicating stubborn warts and it has now been adopted more widely into clinical practice. Logically, the next question to explore is how microwaves work against this viral infection of the epidermis.

Microwaves travel easily through tissues, unlike heat energy from an infra-red source, such as a cautery device, where heat is transmitted by conductance. In contrast to a carbon dioxide laser, for example, energy levels delivered by the Swift microwave device (Emblation Medical Limited, Alloa) are low and not designed to be ablative in nature so there is no tissue vaporisation, hence no smoke, steam or burn.

The temperature is raised to a level that is termed 'heat shock', within the range 41-44˚C, compared with the normal body temperature of 37˚C. At this hyperthermia level the increase in temperature has several effects - hyperthermia is widely acknowledged to provide an anti-tumour response through [5]:

1. Heat dissipation – tumours are more compact and disorganised and so cannot dissipate heat as readily as normal tissue and are therefore more sensitive to heating.
2. Tissue damage or death (apoptosis) to cell membranes and intra-cellular structures.
3. Modulation of a number of immune processes.

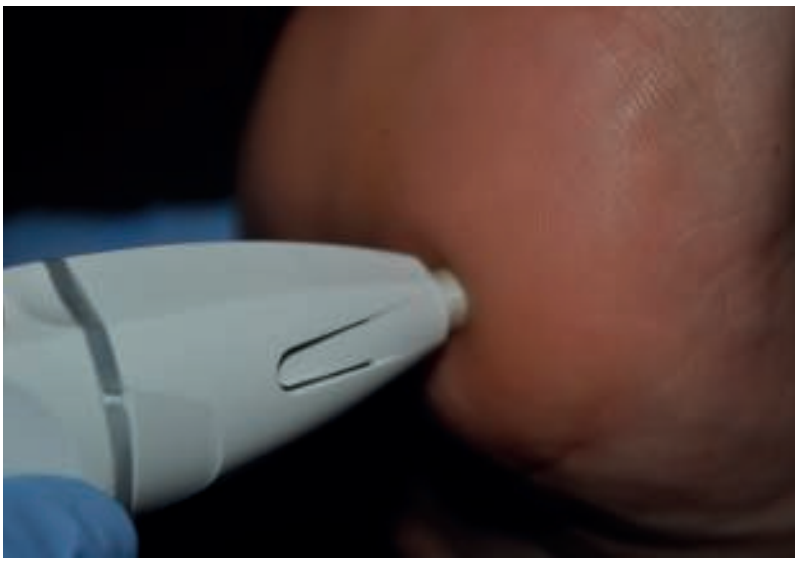
It is this latter point that potentially holds the key to new developments in treatment. Studies have highlighted successful eradication of tumours treated with temperatures in the heat shock temperature range [6]. More specifically, heating has been used to successfully eradicate warts. Huo & colleagues [7]undertook a randomised controlled trial of 54 patients to assess how repeated heating of warts to 44˚C affected resolution. At the end of the study, 54% of the treated group had resolved versus just 12% in the placebo arm. This work also reported that treatment of a single 'target' lesion could promote an immune response that cleared all lesions, resulting in a more tolerable treatment. A downside to this proposed heating treatment regimen that employed infrared energy was the use of 30-minute treatment cycles, which clinically may not be practical.

For normal adaptive immunity to occur in the skin, virally infected tissue must to be taken up by skin dendritic cells and carried to the lymph nodes for priming of CD8+ T cells. Primed T cells migrate from the lymph node and recirculate to the skin where they can then recognise and kill HPV infected skin cells. Warts on the skin are well known for their persistence, suggesting that host immunity is imperfect in dealing with this infection.

A variety of well-established mechanisms for host immune evasion exist, including down-regulation of antigen-processing machinery, and impaired dendritic cell function [8]. For example, previous work has demonstrated that, during HPV skin infection, up-regulation of the PI3-K pathway suppresses anti-HPV responses in Langerhans cells. Inhibition of this pathway increased anti-HPV activity, leading to rapid clearance of HPV [8]. Heating skin has been shown to enhance Langerhans cell migration from the epidermis [9] and, additionally, it has been shown that, by heating tissue, increased temperatures may exert an effect by preventing PI3-K activation, thereby potentiating the immune recognition of HPV infection [10] .

If tissue is exposed to temperatures above 41˚C, cell damage and death is likely. However, cells under stress (such as heating) produce chemicals known as Heat Shock Proteins (HSP). These have evolved to protect cells in extreme stress conditions from cell death. HSPs have a number of functions: as protein chaperones that are involved with the folding, shape regulation and degradation of intracellular proteins [1]. However, their effects on the immune system are of more interest. HSP-70 has been shown to induce the maturation of Langerhans cells and enhance their migration to the lymph nodes. When comparing normal skin to HPV infected skin, it was discovered that the migratory response was more marked in the HPV-infected skin[12]. HSP release also has been shown to stimulate cytokine release from antigen-presenting cells, as well as nitric oxide, chemotactic factors from macrophages and stimulate anti-tumour responses [5].

Other work has discovered that when HPV-infected cells are heated there is a greater release of the natural anti-viral group of cytokines known as interferons. These are important



cell signals that promote immune function. A study by Zhu et al [13] compared the release of interferons by heating virally infected human cells versus uninfected cells, and demonstrated that HPV-infected skin when exposed to 42-45˚C produced larger quantities of interferon than uninfected tissue. It has also been demonstrated that, during hyperthermically induced wart regression, a high level of CD4+ and CD8+ T-lymphocyte infiltration was identified in the treated areas, suggesting that cellular recruitment is enhanced by heat-induced epithelial damage, which is likely to be central to anti-viral immune responses [14].

The above work has demonstrated how research into hyperthermia has shown some positive insights into how raising skin temperature into the 41–45˚C range can bring about cellular changes conducive to resolution, but is there any evidence that such effects are induced by microwave heating? We have previously reported a study of human skin explant sections that were subjected to treatment using the Swift Microwave device and liquid nitrogen to observe for anti-HPV immune activity. Skin keratinocytes normally reside in a non-activated state. However, those from microwave-treated human skin were found to show increased expression of HSP-70 and were able to signal to dendritic cells. Even at a low energy, keratinocyte induced dendritic cell activation induced enhanced cross-presentation of HPV antigens to CD8+ T cells, with consequent interferon-γ production [5.] Interestingly, in liquid nitrogen treated control experiments, similar keratinocyte driven dendritic cell activation was not found.

Most recently the authors demonstrated that microwave treatment of cutaneous warts can be effective. In the first study of its kind, a cohort of 32 adults with refractory warts were treated with a course of microwave therapy using the Swift® device. At the conclusion, the resolution rate was 75.9%, with 41 of the 54 warts reported as resolved [16.]

Taken together, the basic science of microwave effects on skin and clinical responses noted suggest a mechanism for the observed action of microwaves in cutaneous warts, although more research is required to further this knowledge. Our understanding of the molecular mechanisms of hyperthermia provides a strong case for this new technology to be explored further as a local immune response activator therapy. Additionally, it seems likely that there may be many other potential dermatology applications for this exciting technology. ■

Declaration of interests:
Ivan Bristow is a consultant for Emblation Medical Limited.

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CLINICAL TRIAL

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A feasibility study of microwave therapy for precancerous actinic keratosis

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Summary

Background Actinic keratosis (AK) is a common premalignant skin lesion that can progress to cutaneous squamous cell carcinoma (cSCC). Microwave therapy is an established cancer treatment and has been used for plantar viral warts.
Objectives To evaluate the efficacy and feasibility of microwave as a treatment for AK.
Methods Stage I was a dose-setting study, in which seven participants had the dielectric properties of 12 thick and 22 thin AKs assessed for optimization of the microwave dose used for treatment in Stage II. Stage II was a randomized, internally controlled trial evaluating 179 AKs in 11 patients (93 treated, 86 untreated controls) on the scalp/forehead or dorsal hand. Participants received one treatment initially and a repeat treatment to unresolved AKs at week 4. The response was assessed at six visits over 4 months. The primary outcome was partial or complete resolution of the treated AKs.
Results A significantly higher proportion of treated AK areas responded than untreated (90% vs. 15%; $P < 0{\cdot}001$). Thin AKs were more responsive than thick AKs. The site did not affect efficacy. Pain was severe, but brief (80% reported pain lasting 'a few seconds only'). Adverse effects were minimal (erythema, n = 6; flaking, n = 3; itch, n = 3). All participants who would chose microwave therapy over their current treatment cited the shorter discomfort period.
Conclusions Microwave therapy is a portable, safe and effective treatment for AK. An easy-to-deliver, acceptable therapy for AK is attractive as a prevention strategy. While these results are promising, a larger randomized controlled trial is needed against an effective comparator to confirm clinical efficacy and patient acceptability.

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Conflicts of interest
D.N.J. has given lectures for AbbVie and Galderma. P.T.D. reports grants from AbbVie, Gilead and Shire. P.T.D. is a member of the New Drugs Committee of the Scottish Medicines Consortium.

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What is already known about this topic?

- Actinic keratoses (AKs) are common precancerous skin lesions.
- Successful treatment of AK can prevent cutaneous squamous cell carcinoma (cSCC).
- Most topical therapies for AK require repeated application over weeks and drive local skin inflammation, leading to poor compliance.
- An easy-to-deliver and effective treatment for AK, suitable for use in primary care, could reduce cSCC.

What does this study add?

- Microwave therapy is a feasible, effective treatment for AK.
- Ninety per cent of treated AKs showed full or partial resolution at 120 days post-treatment.

- Microwave therapy was painful, but the pain was short-lived (seconds) and this short discomfort period was cited as the main reason that microwave was preferred to their current treatment.

Actinic keratosis (AK) is a common precancerous skin lesion found on light-exposed sites in older fair-skinned individuals with prevalence rates of 23·5% in the Dutch population over 50 years of age.[1] AKs are precursors to cutaneous squamous cell carcinoma (cSCC), which has doubled in incidence in a decade due to ageing populations and increased ultraviolet radiation exposure.[2] The individual risk of progression to cSCC is low,[3] but 65% of cSCCs on the head and neck arise from AK.[4] A double-blind, randomized clinical trial (RCT) of 5% fluorouracil cream (5-FU) showed a 75% risk reduction for development of cSCC in the year following treatment [95% confidence interval (CI) 35–91%; $P < 0{\cdot}002$].[5] This pivotal study suggests that annual treatment of AK should reduce the incidence of cSCC. Multiple field treatments for AK exist, such as 5-FU, imiquimod 5% cream, diclofenac 3% gel, photodynamic therapy (PDT) and lesion-directed therapy like liquid nitrogen (cryotherapy).[6] Many AK treatments require dedicated application over weeks and drive significant inflammation. Furthermore, many AK sufferers are elderly and would find compliance easier with a lesion-directed treatment.

Microwave therapies are established within oncology for ablative treatment of internal malignancies.[7,8] Microwave energy has shown promise in the treatment of recalcitrant plantar viral warts.[9] This study used a CE-marked microwave medical device (Swift® Microwave Tissue Ablation System, Emblation Ltd, Alloa, UK). The applicator of the Swift® device delivers microwave energy to the skin at a diameter up to 6 mm and depth of 2–6 mm depending on dosage. The electromagnetic waves excite water molecules, driving localized hyperthermia[10] and accelerated chemical kinetics.[11] Depending on dose, the treatment can have an ablative destructive or subablative nondestructive effect.

Here we report a first-in-human, two-stage feasibility study of microwave therapy for the treatment of AK on the bald scalp, forehead or dorsal hand.

For Stage I, the objective was to determine the dielectric properties of AK for optimization of the Swift® device microwave parameters to deliver a subablative dose of energy.

Stage II was a single-site, randomized, internally controlled trial to evaluate the efficacy, long-term resolution, safety and feasibility of microwave as a treatment.

Materials and methods

Study design and participants

This randomized, internally controlled, feasibility study of microwave therapy for the treatment of AK (NCT03483935) was conducted at Ninewells Hospital & Medical School, Dundee, UK, from January 2018 until April 2019. The study was co-sponsored by the University of Dundee and NHS Tayside (approved December 2017) and was reviewed and approved by the East of Scotland Research Ethics Service (18/ES/0008, January 2018). Patients with AKs on the forehead, bald scalp or dorsal hands were recruited from the dermatology department, NHS Tayside. All participants provided written informed consent.

Inclusion criteria were age over 18 years with a minimum of six AKs on both the right and left side of the forehead/scalp or dorsal hand, able to give informed consent and perform study assessments. Exclusion criteria were AKs sited on the lip or ear, confluent AKs with field change, implantable cardioverter-defibrillator, pacemaker or other implantable device, metal implants at the site of microwave treatment, known intolerance to microwave, unstable comorbidities (including cardiovascular disease, active malignancy, inflammatory arthritis) or participation in another interventional study.

Sample-size calculations were based on 100 AKs (50 treated; 50 untreated, mapped and followed), with on average 10 per participant. In a paired analysis with McNemar's test, the power is 80% to detect a difference in proportion > 25% complete or partial resolution of 0·33. Repeated measures of AKs over six visits will give 300 paired measurements. Even with a smaller mean number of visits (i.e. three), the number of pairs is increased by the inflation factor to 55 assuming intraclass correlation coefficient of 0·05. Hence, with a mean of three visits (i.e. 150 paired measurements), power would be 80% to detect a difference of 0·2 between treated and untreated in proportion > 25% complete or partial resolution.

Microwave dose

For Stage I, seven participants had the relative permittivity, conductivity and loss tangent of their AK measured. They did not receive any microwave dose. A median of five measurements per patient were taken, of which 22 (65%) were for thin AKs (Olsen grade 1 or 2) and 12 (35%) were for thick AKs (Olsen grade 3).[12] The data allowed calculation of the energy required to raise the tissue temperature into the subablative region.[13] Subablative doses are generally considered to be below 50 °C.[13] A microwave treatment dose of 5 watts (5W) delivered for 3 s and repeated three times with 20-s gaps was chosen.

When the 5W dose was delivered to the first two participants, it caused severe pain and some ulceration/scabbing, suggesting it was ablative rather than subablative. Modelling performed by Emblation, the manufacturer of Swift®, using data from the permittivity study and a finite element solution

of the bioheat transfer equation suggested that 4W for hyperkeratotic 'thick' AK (Olsen grade 3) and 3W for nonhyperkeratotic 'thin' AK (Olsen grades 1 and 2) would still provide a therapeutic subablative tissue temperature, with the benefit of being more tolerable (Table S1; see Supporting Information).[14] Consequently, the protocol was amended to reflect this reduction in dose. The protocol change was submitted as a substantial amendment and approved by the East of Scotland Research Ethics Service prior to continuing the study.

Study assessments

At the screening visit, participants consenting to Stage II had the treatment site (scalp/forehead or hands) chosen by the Chief Investigator based on a clinical decision. AKs were mapped using an acetate grid and photographed using an agreed protocol to aid assessments at later visits. Participants underwent a general examination to exclude significant comorbidities and coincidental skin cancer. Participants were randomized to treatment to the left or right side. The randomization system used was TRuST, a good clinical practice-compliant Tayside Clinical Trials Unit Interactive Web Response System. No stratification or minimization was used. One AK on the treatment side was preselected at screening for biopsy at visit 4. The treatment side received microwave therapy, with mapped AKs on the contralateral side observed as untreated controls. The probe was placed in the centre of the AK for each treatment and AKs larger than the treatment probe were not excluded as it was unclear whether benefit might extend to adjacent areas of AKs through local inflammatory or immunological effects. We wished to test whether AKs larger than the applicator tip would resolve completely or only partially. Participants were asked to rate their pain immediately following treatment and 30 min later.

Participants attended for six follow-up visits at 1, 2, 4, 6, 8 and 16 weeks post-microwave treatment. At each visit AKs were assessed by the Chief Investigator or delegate and scored as completely resolved, partially resolved or unchanged. Participants were asked about local adverse events (itching, stinging, soreness, redness, flaking, ulceration, pus) and whether these were mild or severe. Photographs were taken of treated and control AKs at each visit to aid assessment. There were three telephone follow-ups on weeks 3, 5 and 7 to assess adverse events following treatment and biopsy.

A pre-assigned, treated AK was biopsied (4-mm punch biopsy) at 2 weeks post-treatment (visit 4) for histology and transcriptome studies.

At week 4, there was the option to repeat treatment to any previously treated but unresolved AK.

The final visit and AK assessment took place at 4 months (day 120). Participants were asked to complete a self-assessed health index about their experience of microwave therapy.

Statistical analysis

The primary outcome, resolution of the treated area of the AK lesion, was predetermined as either partial (resolution of the area covered by the microwave probe, but with a rim of persistent AK) or full resolution (complete resolution of the entire AK) over all time periods. Response was assessed at visits 3 (day 8), 4 (day 15), 6 (day 28), 8 (day 42), 10 (day 60) and 11 (day 120). Mixed-effects logistic regression models analysed the effect of microwave therapy with random effects for participant and visit ($\leq$ 6 per participant). Each visit was analysed as a categorical variable as they were spaced unequally in time. Variables representing sex, age, skin site (hand/scalp) and AK subtype (thick/thin) are included as covariates.

The secondary outcome of long-term response was assessed using data from visits 10 and 11 (days 60 and 120) only. Again, nonlinear models were utilized with random effects for participant and visit ($\leq$ 2 per participant) and covariates for sex, age, skin site (hand/scalp) and AK subtype (thick/thin).

Pain during and following treatment were secondary outcomes. SAS Enterprise Guide software (version 6·1, SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Study participants

Eleven participants, seven male and four female, gave informed consented and were randomized to Stage II. The Consort flow diagram is illustrated in Figure 1. Participant demographics and cancer history are shown in Table 1. Ten of 11 (91%) participants had received prior treatments with both cryotherapy and 5-FU cream. Additional treatments in order of frequency were 3% diclofenac gel (Solaraze) (64%), 5% imiquimod cream (Aldara) (55%), ingenol mebutate gel (Picato) (45%), surgery (45%), PDT (36%) and 5-FU 0·5% and salicylic acid 10% topical solution (Actikerall) (27%) as detailed in Table S2 (see Supporting Information).

Microwave dose

Following permittivity studies (Stage I), the microwave dose was chosen and delivered as described in Materials and methods. The first two participants in Stage II received a 5W dose for 3 s repeated three times to each treated AK, but the subsequent nine participants received 3W 3-s doses to thin AKs (Olsen grades 1 & 2) and 4W 3-s doses to thick AKs (Olsen grade 3).

Treatments and biopsies

Eleven participants were randomized to the RCT and 179 AKs (93 treated, 86 untreated) assessed (Figure 1). All participants completed treatment as planned and at follow-up visit 10 (day 60) one participant's treated AKs could not be assessed due to an unrelated hospital admission (the hand was bandaged for an *in situ* cannula). Ten participants underwent a second treatment on day 28 (visit 6), with 51 of 93 (55%) treated AKs receiving a second treatment. One of the participants who had

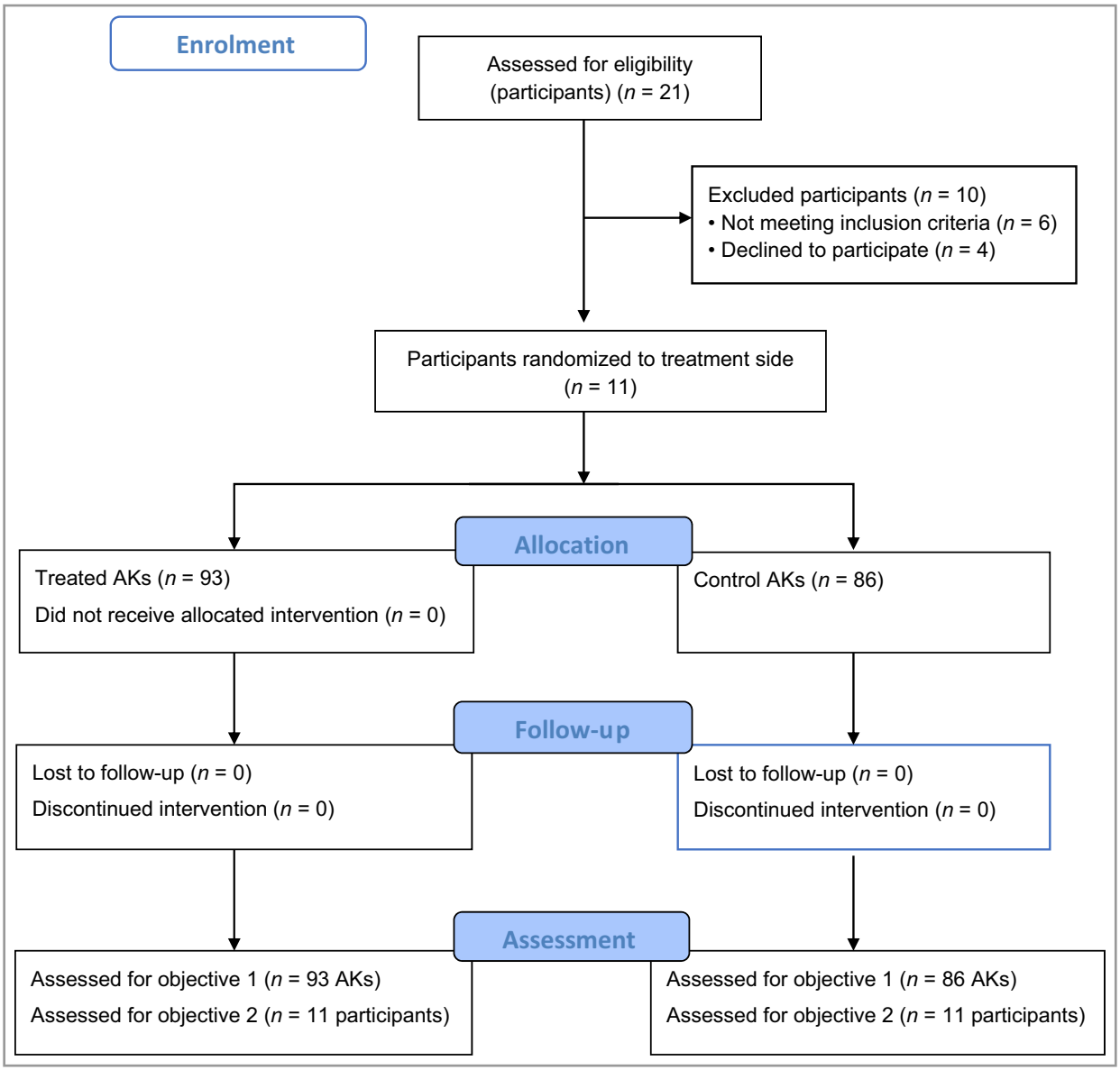

Figure 1 CONSORT diagram.

Table 1 Baseline characteristics for 11 participants

Variable	Statistic/status	Summary
Age (years)	n	11
	Mean (SD)	78 (6)
	Median	78
	Range	62–88
Sex	Female	4 (36%)
	Male	7 (64%)
History of skin cancer	Yes	10 (91%)
	No	1 (9%)
History of other cancer	Yes	0 (0%)
	No	11 (100%)

received the 5W dose declined repeat treatment due to pain. All biopsies of pre-assigned AKs were undertaken at day 15, 2 weeks after the first treatment.

Biopsies of AKs treated with 5W (n = 2) showed dermal fibrosis, mixed acute and chronic inflammatory infiltrate, and some reactive squamous metaplasia of eccrine ducts. Any epidermal dysplasia was mild. Six of the remaining nine AK biopsies showed some inflammation with mild-to-moderate epidermal dysplasia in the majority, although three of nine noted moderate-to-severe epidermal dysplasia consistent with persistent AK.

Effectiveness of treatment

Overall response rates (including both partial and complete resolution) for treated AKs were 78% at visit 3 (day 8), rising to 90% at visit 11 (day 120) (Table 2, Figure 2), compared with 2% at visit 3 and 15% at visit 11 for untreated AK. The results of a nonlinear repeated measures model of resolution

found a significantly higher proportion of AKs treated with microwave therapy to have fully or partially resolved compared with untreated control AKs (odds ratio 154, 95% CI 75–317, $P < 0{\cdot}001$, Table 3). The magnitude of the odds ratio reflects the sustained resolution of treated AKs across all visits (Table 2, Figure 2). The photographs in Figure 3 show examples of partial and complete resolution.

The type of AK (thick vs. thin) was associated with response (Table 3, Figure 4). Thin AKs had a higher response rate than thick AKs, but similarly, in the untreated group, thin AKs were more likely to resolve spontaneously (Figure 4).

Participant-reported pain and safety

Most participants reported 'moderate' or 'severe' pain during treatment and all participants reported no pain after 30 min (Table 4). Eighty per cent of participants reported pain lasting a few seconds only; 20% reported pain lasting up to 5 min. Redness (n = 6), flaking (n = 3) and itching (n = 3) were reported as adverse events. There were no unexpected or serious side-effects. Most participants preferred microwave or had no preference when comparing their experience of microwave with previous treatments (Table 5).

Table 2 Summary for primary endpoint by visit for AK lesions

	Treated			Not treated		
Visit	Lesions, n	n (%)	Resolution	Lesions, n	n (%)	Resolution
Visit 3 (day 8)	93	73 (78)	4 CR, 69 PR	86	2 (2)	1 CR, 1 PR
Visit 4 (day 15)	93	73 (78)	7 CR, 66 PR	86	7 (8)	1 CR, 6 PR
Visit 6 (day 28)	93	86 (92)	20 CR, 66 PR	86	9 (10)	2 CR, 7 PR
Visit 8 (day 42)	93	86 (92)	23 CR, 63 PR	86	9 (10)	3 CR, 6 PR
Visit 10 (day 60)[a]	84	81 (96)	41 CR, 40 PR	86	11 (13)	5 CR, 6 PR
Visit 11 (day 120)	93	84 (90)	39 CR, 45 PR	86	13 (15)	6 CR, 7 PR

[a]The clinical investigator was unable to assess nine treated AKs on one participant at visit 10 due to the participants' hand being cannulated for other medical treatment during an unrelated inpatient admission. CR, complete resolution; PR, partial resolution

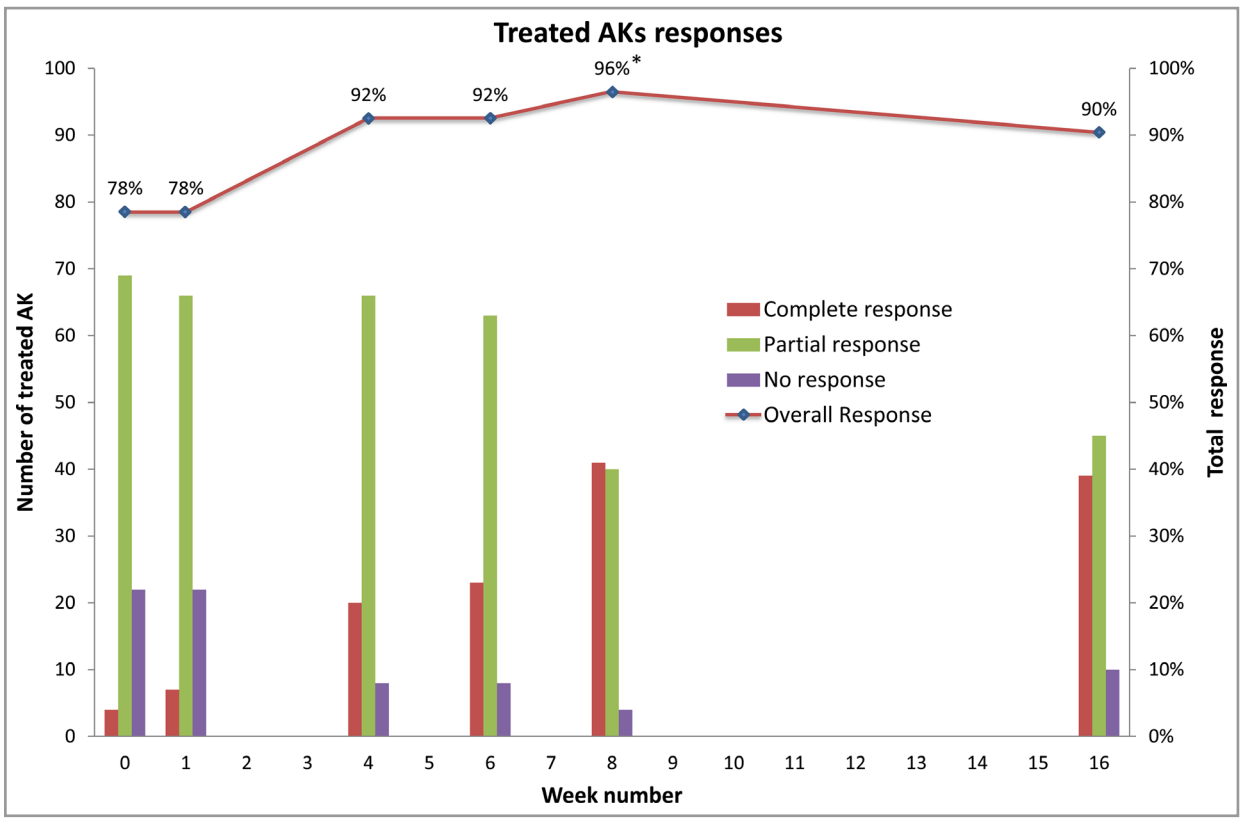

Figure 2 Percentage of treated actinic keratoses (AKs) that responded by week.

Table 3 Odds ratios (OR) from the mixed model for actinic keratosis response

Variable	OR	95% CI	P-value
Treatment vs. placebo	154	75–317	< 0·001
Visit			< 0·001
Visit (3 vs. 11)	0·25	0·1–0·58	
Visit (4 vs. 11)	0·33	0·14–0·75	
Visit (6 vs. 11)	0·87	0·42–1·78	
Visit (8 vs. 11)	0·87	0·42–1·79	
Visit (10 vs. 11)	1·25	0·65–2·42	
Age (+ 1 year)	1·03	0·97–1·08	0·33
Sex: female (ref, male)	0·56	0·26–1·21	0·14
Scalp site (ref, hand)	1·68	0·78–3·61	0·18
Thin subtype (ref, thick)	3·47	1·8–6·66	< 0·001

CI, confidence interval

Discussion

This first-in-human study suggests that microwave therapy might be a promising treatment for AK, with 90% of AKs showing resolution of the treated area at 120 days post treatment (Table 2). This was highly significant ($P < 0·001$) and was more effective for thin than for thick AKs ($P < 0·001$), as has been noted with most other therapies, including PDT.[15,16] Our study included hyperkeratotic AKs and acral sites, both of which are associated with higher rates of treatment failure.[6,17]

AKs larger than the microwave probe diameter were not excluded in this study. Many larger AKs demonstrated complete resolution of the central area under the applicator tip, but with a rim of persistent AK outside this treatment area and these lesions were recorded as a partial response despite resolution of the treated area. Therefore, rates of complete

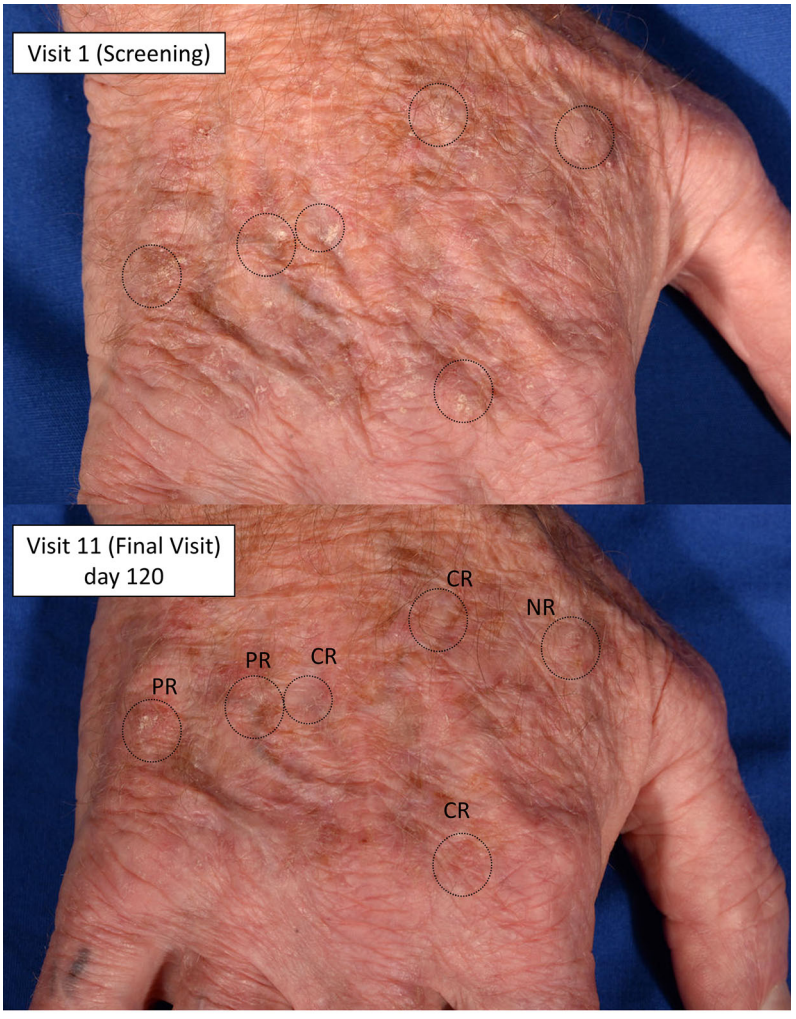

Figure 3 Photographs at visit 1 (screening visit) and visit 11 (day 120) show improvement following microwave therapy for selected hyperkeratotic actinic keratoses [marked by circles (n = 6)]. CR, complete resolution; NR, no resolution PR, partial resolution.

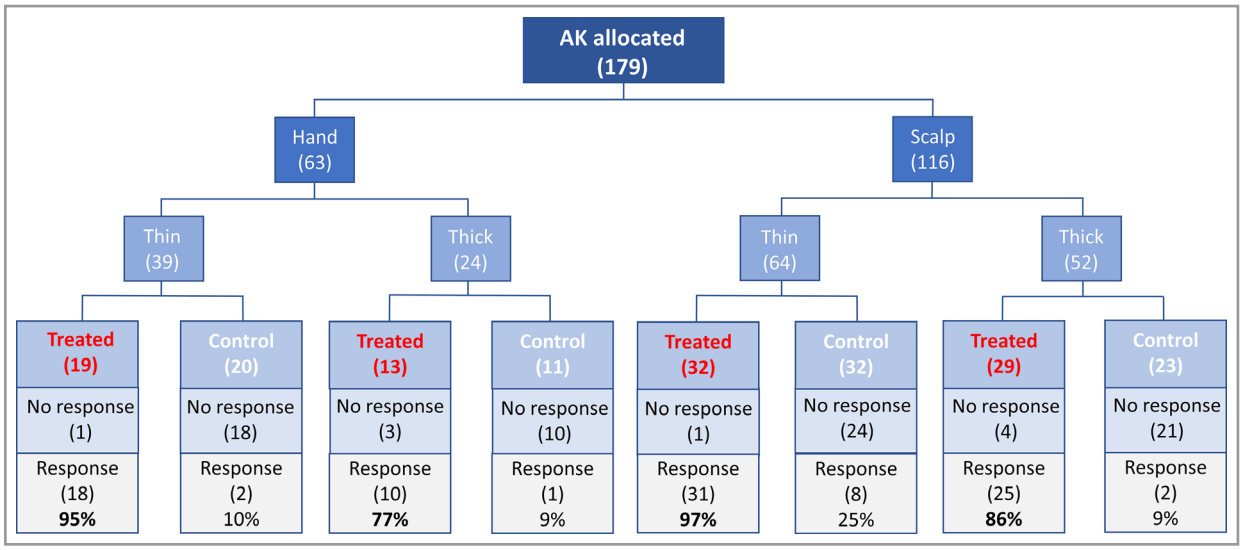

Figure 4 Summary tree.

Table 4 Summary of pain during treatment

Variable	Visit 2 participants, n (%)	Visit 6 participants, n (%)
Pain during treatment		
Mild	7 (8)	5 (10)
Moderate	32 (34)	27 (53)
Severe	54 (58)	19 (37)
Duration of pain		
Few seconds	7 (64)	8 (80)
Up to 5 min	2 (18)	2 (20)
Up to 10 min	1 (9)	0 (0)
Up to 20 min	1 (9)	0 (0)
Still sore at 30 min	0 (0)	0 (0)

Table 5 Patient experience

	Week 10	Week 11
Choice of another AK treatment		
Prior treatment	1	2
Microwave	4	6
No preference	6	3
Reason for choice[a]		
Less pain	1	1
Shorter discomfort period	3	6
Fewer side-effects	3	4
Other	2	1
Pain if second treatment		
More painful	1	0
Less painful	5	5
No difference	4	5
Not applicable	1	1
Worry about treatment		
Yes	0	1
No	11	10

AK, actinic keratosis

[a]Not all participants gave a reason for their choice and some participants chose more than one reason for their choice

resolution in this study appear to be relatively modest, with 42% of AKs showing complete resolution at 120-day follow-up. In future studies, adopting a stepwise overlapping delivery of treatment across the whole surface of the AK might lead to higher rates of complete resolution. This approach has been used successfully for plantar warts.[9] Seven per cent of untreated AKs had spontaneously resolved by visit 11, which is similar to that reported in other studies[18] and demonstrates the importance of an internal control.

The participants in this study were representative of the patient population with AK, with a median age of 78 years and majority male (64%). An increased prevalence of AK with advancing age and male sex has been noted in both primary[19] and secondary care.[20] Importantly, there was no difference in response with sex, age or skin site. In contrast, many alternative therapies have demonstrated reduced efficacy on acral sites.[6,17]

One week after initial treatment (visit 3), 78% of AKs showed a response, which rose to 92% by 4 weeks (visit 6), which may suggest that induction of an immune response promotes clearance. A similar effect was seen with treatment to plantar warts.[9] This implies that there may be a possibility of 'field' benefit with microwave therapy and in this study, as demonstrated in Figure 3, the post-treatment appearance did show a general improvement in addition to specific resolution (partial or complete) of individual AKs. Nonetheless, microwave therapy should be considered a lesion-directed therapy rather than a field-directed therapy and any subsequent examination in a head-to-head comparison with current AK therapies should include a cryotherapy arm as well as a topical 5-FU or imiquimod arm.

The main side-effect was pain. The first two participants, treated with 5W doses, found the treatment very painful and one participant declined repeat treatment at 4 weeks. This higher dose appears more efficacious with 100% of AKs showing a response to the 5W dose compared with 88% with the 3W or 4W dose (Table S3; see Supporting Information). All participants were included in the overall statistical assessment. The subsequent lower doses (3W or 4W), delivered after an amendment to the protocol, were well tolerated. Participants described pain as 'minimal' initially, but 'very painful' for the final 1 s of treatment. While 58% of participants described pain as severe at the first treatment, this reduced to 37% with the second treatment, and most (80%) reported the pain as lasting a few seconds only (Table 4). This reduction may be due to greater expectation with repeat treatment. All participants eligible for the study completed it and none of the participants who received the lower microwave dose with the revised protocol declined a second treatment, suggesting that this was tolerable, despite treatment of up to 10 AKs per treatment. Rarely did pain last longer than 5 min (Table 4) and severe pain never lasted more than a few seconds. This short duration undoubtedly makes the treatment more tolerable. When surveyed about their patient experience on day 120, six patients would choose microwave treatment over their current AK therapy, and all cited a shorter discomfort period as a reason (Table 5). Pain is common with existing treatments such as cryotherapy and PDT; however, patients often accept this if a treatment is effective.[21]

Other adverse events following microwave treatment were minimal [erythema (n = 6), flaking (n = 3) and itch (n = 3)]. This contrasts favourably with treatments like 5-FU or 5% imiquimod cream, which cause significant inflammation including swelling, erosions, crusting and blistering.[22] Diclofenac 3% in hyaluronic acid gel causes less severe local skin reactions[23] and is a treatment favoured by general practice in the UK.[24,25] However, its long treatment duration (3 months) may reduce compliance.

There were study limitations. This was a small study with 11 participants, but analysis was per AK and 179 AKs were assessed, increasing the power. Participants were recruited from secondary care so had relatively severe AK. Sufficient 'thick' and 'thin' AKs were treated to analyse these subgroups independently. While the side to receive treatment was randomized, the assessors were not blinded. Blinding was not feasible as only two clinicians were involved in both treatment delivery and follow-up. Furthermore, erythema from the treatment and, at later visits, the biopsy scar, would reveal the treatment side. As a first-in-human study, the effects of treatment on AKs were not known. As such, both partial and complete resolution of individual AKs were assessed and we have reported the response rates by complete, partial or none in Table 2 and Figure 2. Due to the lack of overlapping or stepwise treatment over the entire surface of larger AKs, this study may underestimate the potential for complete resolution. There was minimal missing data.

The microwave device used is portable and safe and does not require the impractical storage infrastructure of cryotherapy. Minimal training is needed and multiple lesions can be treated in a single session. These factors make it particularly suitable for primary care where AKs are prevalent. Of 2844 consecutive patients enrolling with a general practitioner in Switzerland, 23% had AK.[19] Given the mounting evidence that treatment of AK can prevent cSCC,[5] a treatment that can be delivered in primary care, possibly when the patient is attending for another reason, may be effective at reducing the burden of cSCC. While these results are promising, a larger RCT is needed against an effective lesion-directed comparator such as cryotherapy, as well as a field treatment, to confirm clinical efficacy and patient acceptability.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Results of Stage I modelling.

Table S2 Previous therapies and preference by dose.

Table S3 Summary for primary endpoint by treatment protocol.

Microwave hyperthermia represses human papillomavirus oncoprotein activity and induces cell death due to cell stress in 3D tissue models of anogenital precancers and cancers

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Summary

Background Hyperthermia is a well-accepted cancer therapy. Microwaves provide a very precise, targeted means of hyperthermia and are currently used to treat plantar warts caused by cutaneous-infective human papillomaviruses (HPVs). Other HPV genotypes infecting the anogenital mucosa cause genital warts or preneoplastic lesions or cervical cancer. Effective, non-ablative therapies for these morbid HPV-associated lesions are lacking.

Methods The molecular consequences of microwave treatment were investigated in *in vitro* cultured three-dimensional HPV-positive cervical tumour tissues, and tissues formed from HPV-infected normal immortalised keratinocytes. Microwave energy delivery to tissues was quantified. Quantitative reverse transcriptase PCR was used to quantify mRNA expression. Immunohistochemistry and fluorescence immunostaining was used to assess protein expression.

Findings Microwave energy deposition induced sustained, localised cell death at the treatment site. There was a downregulation in levels of HPV oncoproteins E6 and E7 alongside a reduction in cellular growth/proliferation and induction of apoptosis/autophagy. HSP70 expression confirmed hyperthermia, concomitant with induction of translational stress.

Interpretation The data suggest that microwave treatment inhibits tumour cell proliferation and allows the natural apoptosis of HPV-infected cells to resume. Precision microwave delivery presents a potential new treatment for treating HPV-positive anogenital precancerous lesions and cancers.

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Keywords: Human papillomavirus; Cervical disease; Microwave hyperthermia; Cell stress response; HSP70

Introduction

Around 40 human papillomaviruses (HPVs) infect the anogenital region.[1] Some cause genital warts but others ("high-risk" (HR-HPV)) cause >99% of cervical cancers and between 40 and 90% of other anogenital cancers in men (anal, perineum, penile) and women (anal, vulvar, vaginal, perineum).[2,3] Incidence of these cancers is increasing.[3] Treatment of cervical precancers, or cervical intraepithelial neoplasia (CIN), is "loop" excision (LLETZ) of the cervix[4] or "cold coagulation", cauterisation with a heated probe.[5,6] These treatments are effective but invasive and painful, and for LLETZ, often with subsequent bleeding and a two to three-fold increase in preterm birth.[7] Treatment of other anogenital precancers also involves excision.[4] Those with HR-HPV-associated anal or cervical precancers are at risk of multifocal anogenital disease,[8] which is challenging and costly to treat with significant morbidity due to treatment and the need for a multidisciplinary clinical team for disease management.[4] In all cases, diseased tissue can be missed. A new, less invasive and painful method for treating HPV-associated anogenital disease and

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Research in context

Evidence before this study

"High risk" human papillomaviruses (HR-HPVs), cause anogenital lesions such as genital warts, anal, penile, cervical, vulval and vaginal intraepithelial neoplasia (precancers) and cancers. Incidence of most of these diseases and cancers is increasing worldwide. Laser, or electrical ablation, or "cold coagulation" (use of a hot probe to burn away lesions) are current options for treating cervical precancers and cancers. Treatment for other anogenital cancers is surgical excision. Precancerous lesions or warts may also be excised and/or treated with imiquimod (immune response modifier) or cidofovir (viral replication inhibitor). These drugs can cause inflammatory reactions, which are not well-tolerated, and lesions can recur. Women with HR-HPV-associated anal or cervical precancers are at risk of multifocal anogenital disease. This is challenging and costly to treat due to multiple lesions, significant treatment-related morbidity and sometimes the need for extensive reconstructive surgery.

Localised hyperthermia (heating to fever temperature range) has proved useful in cancer therapy to elicit anti-tumour effects, including immune activation. Microwaves can deliver mild hyperthermia in a controlled, transient, and linear fashion. Recently, microwaves were used to treat recalcitrant verrucas, caused by cutaneous-infective HPVs. Microwaving produced a 76% clearance rate, compared to, at best, 33% by cryotherapy. Data suggested that the effect was mediated through enhanced immune activation. A recent clinical trial also showed efficacy of microwaving against actinic keratoses (HPV-associated precancerous rough skin patches due to years of sun exposure). Although these dermatological diseases are caused by non-anogenital HPVs, the close genetic and biological similarity of different types of HPV suggests that heat treatment by microwaving could clear other HPV-associated diseases. Extension of therapeutic use to anogenital disease could deliver a better treatment option than current approaches. Moreover, with further development, the hyperthermia approach could be broadly applicable to other types of precancers or carcinomas *in situ*.

Added value of this study

This study reveals the molecular effects of microwave energy on HPV-infected epithelial tissues *in vitro*. The results show the precision of microwave delivery to tissues. Microwave energy resulted in a sustained reversal of the tumourigenic phenotype; a decrease in cellular proliferation and an increase in apoptosis/autophagy due to induction of a cellular heat shock and translational stress response. Importantly, the treatment reduced expression of the HPV oncoproteins E6 and E7, whose increased expression underlies progression of HPV-associated cancers.

Implications of all the available evidence

All the evidence indicates that microwave treatment using a low heat energy precision device could be a novel therapy for anogenital disease, particularly for those cases such as HPV-associated anogenital multifocal disease that normally prove difficult to treat effectively. Current treatment for anogenital disease can cause sustained bleeding or inflammation and diseased tissue can be missed. Heat treatment through microwaving could provide better tissue coverage and be more acceptable and better tolerated than existing strategies. The mild nature of the procedure would mean that sequential treatments could be spaced more closely and could save clinic time and resources.

cancers could prove more acceptable and better tolerated by patients than current procedures and would save time and resources for clinicians and health care systems.

Hyperthermia, especially in combination with radio- or chemotherapies, is a well-known treatment for cancers such as cervical, melanoma and breast cancer.[9–11] Sustained temperatures above 40 °C result in membrane disruption, DNA and protein damage and cell cycle arrest followed by necrosis or apoptosis.[12] Heating causes cytotoxicity through impaired functions of proteins involved in key cellular processes such as DNA replication and can induce anti-tumour immune responses.[10]

Microwaves efficiently deliver mild heat energy (<50 °C) in a highly controlled, transient localised, linear manner.[13] They produce non-ionising radiation, so do not induce DNA damage. The delivery of microwaves to tissues reaches a depth of up to 6 mm depending on the generator frequency and energy level used and there is little lateral spread. Microwaves generate heat by a process known as dielectric hysteresis. Water molecules in tissues try to align with an electromagnetic field of rapidly alternating polarity generated by microwaves. This results in rapid spinning of water molecules in the tissue and this energy is converted to rapid, highly localised heat generation. Thus, heat generated by microwaves is quite different from heat generated by other means particularly since it involves heating from the inside to the outer surface.[14] This method of heating has been used previously to treat cancers such as liver cancer.[15]

A CE-marked portable medical device which delivers microwaves through a 6.7 mm contact site is currently approved for use in in the fields of podiatry and dermatology in the treatment of plantar warts (verrucas).[16] Plantar warts are caused by infection with cutaneous-infective human papillomaviruses (HPVs), e.g. HPV genotype 1.[17] Microwave treatment of plantar warts resulted in the shrinkage and clearance of lesions

without significant inflammation, visible tissue damage or scarring and an antiviral immune response was generated.[18] There was a final resolution rate of 75.9% compared to 23–33% for standard cryotherapy treatments. Low pain scores were reported which decreased as treatment plans proceeded.[16]

A recent UK clinical trial (NCT03483935) showed microwave therapy to be a safe and effective therapy for actinic keratoses,[19] which may have an underlying beta-HPV aetiology.[20] Although these types of cutaneous lesions are caused by non-anogenital-infective HPVs, the close genetic and biological similarity of different types of HPV suggests that heat treatment by microwave energy could clear other HPV-associated diseases.

In this study we tested if localised microwave hyperthermia could have a therapeutic effect against HPV-associated anogenital precancerous and cancerous tissues. For the cancer model we used SiHa cells, which are HPV16-positive cervical epithelial cells derived from a grade II squamous cell carcinoma.[21] The precancer model was normal immortalised foreskin keratinocytes expressing the HPV16 genome (NIKS16) or the HPV18 genome (NIKS18).[22,23] To mimic *in vivo* tissue, cells were grown in three dimensional organotypic raft cultures.[24] We determined the energy settings required to elevate tissue temperature to 45–48 °C. Application of microwaves caused local tissue heating and there was reduced cell proliferation at adjacent sites concomitant with expression of apoptosis markers. HSP70 was induced indicating successful temperature elevation. Microwave-treated cells displayed a translation stress response. Importantly, in SiHa cancer cells HPV oncoprotein expression was reduced. Correlating with this, levels of the apoptosis regulator p53, normally repressed by HPV E6[25] and levels of Rb, normally a degradation target of HPV E7[26] were significantly increased in the tissues upon microwave treatment. Taken together the data suggest that microwave treatment of HPV-positive tumour tissue reversed the tumour phenotype and induced cell stress leading to inhibition of tissue growth and induction of apoptosis.

Methods

Cell lines

J2 3T3 fibroblasts were grown in Dulbecco's modified eagle medium (DMEM; Life Technologies) supplemented with 10% donor calf serum, 2 mM L-Glutamine (Life Technologies), 100 units/ml penicillin and 100 µg/ml streptomycin (PenStrep, Life Technologies).[24] NIKS16 (RRID:CVCL_B0UM) and NIKS18 (RRID:CVCL_B0UP) cells were grown on 3T3 fibroblast feed layers in E-medium as previously described.[27] SiHa cells (IZSLER Cat# BS TCL 112, RRID:CVCL_0032)[21] were grown in DMEM supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (PenStrep, Life Technologies). All cells were cultured at 37 °C in 5% carbon dioxide (CO_2).

Cell line validation

All cell lines were routinely tested for mycoplasma. SiHa, NIKS16 and NIKS18 cell lines were validated by routinely comparing morphology from batch-to-batch growth in 2D and 3D tissue cultures and by quantifying HPV DNA genome numbers, and E6E7 mRNA expression levels by qRT-PCR. Expression of viral life cycle markers E4 and L1 proteins was also verified in each batch of NIKS16 or NIKS18 cells to ensure they supported HPV replication.[28] NIKS16 and NIKS 18 cell lines were used at passage <12 to avoid HPV genome integration. Tissue distribution of proliferation and differentiation markers was validated as unchanged in every experiment by examining in-tissue expression of MCM2, Ki67, Involucrin and Keratin 10 in untreated tissues by immunofluorescence and western blotting.

Antibodies

Immunohistochemistry (IHC) antibodies were: active caspase 3 (R and D Systems Cat# AF835, RRID:AB_2243952, 1:1000), mini chromosome maintenance protein 2 (Abcam Cat# ab31159, RRID:AB_881276, 1:100), Ki67 (Agilent Cat# M7240, RRID:AB_2142367, 1:200), p53 (Abcam Cat# ab1101, RRID:AB_297667, 1:3000), Rb (Cell Signaling Technology Cat# 9309, RRID:AB_823629, 1:1600) and heat shock protein 70 (HSP70) (Sigma–Aldrich Cat# H5147, RRID:AB_477057, 1:3200). Immunofluorescence antibodies were used at the following dilutions: HPV E6 (Euromedex, #6F4, 1:200), HPV E7 (Thermo Fisher Scientific Cat# 28-0006, RRID:AB_2533057), 1:50), cleaved caspase 3 (R and D Systems Cat# AF835, RRID:AB_2243952, 0.3 µg/ml), Ki67 (Abcam Cat# ab206633, RRID:AB_2861195, 1:1000) GTPase-activating protein SH3 domain-binding protein (G3BP) ((Abcam Cat# ab56574, RRID:AB_941699, 1:250) and poly (A) binding protein C1 (PABPC1) (Abcam Cat# ab21060, RRID:AB_777008, 1:1000), Keratin 10 ((Abcam Cat# ab9026, RRID:AB_306950, 1:200), MCM2 (Abcam Cat# ab31159, RRID:AB_881276; 1:100), involucrin (Sigma–Aldrich Cat# I9018, RRID:AB_477129, 1:200), LC3B (Novus Cat# NB100-2220, RRID:AB_10003146, 1:200), p62 (MBL International Cat# PM045B, RRID:AB_1953130: 1:500), and HSP70 (Sigma–Aldrich Cat# H5147, RRID:AB_477057), 1:200).

Antibody validation

Antibody validation was carried out by examining protein detection at a range of antibody concentrations and quantifying reproducibility between experiments. Specificity was examined by carrying out western blotting to ensure that a single band of the correct molecular mass was obtained with each antibody. Secondary antibody only controls were included in every experiment. E6 antibody is not suitable for western blotting and so was

validated by comparing reactivity in immunofluorescence in HPV-negative C33a cells and C33a cells stably expressing E6.[29] Other antibodies were verified by comparing protein depletion following siRNA knock down by western blotting. The same batch of each antibody was used throughout.

3D organotypic raft tissue cultures

3D cultures were performed exactly as described,[24] grown on metal grids and incubated at 37 °C with 5% CO_2 at the air liquid interface with E-medium for 14 days to allow tissue growth. E-medium was composed of a three to one ratio of DMEM: F12 medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (PenStrep, Life Technologies) 2 mM L-glutamine, 180 μM adenine, 5 μg/ml transferrin, 5 μg/ml insulin, 0.4 mg/ml hydrocortisone, 0.1 nM cholera toxin and 0.2 ng/ml epidermal growth factor (EGF).[24]

Microwave treatment of 3D tissue cultures

3D *in vitro* grown tissues were lifted from the grids and placed cell side down onto the lid of a 10 cm dish that had been pre-incubated at 37 °C (Supplementary Figure S1). The dish was closed, and the microwave probe was then positioned underneath the plastic lid, facing the cell layers of the tissue and set to deliver 10 W of power for 10 s (unless otherwise specified). Tissues were either fixed immediately in formalin overnight or returned to the air-liquid interface and incubated for up to 144 h at 37 °C in 5% CO_2 prior to fixation. "Mock treated" tissues underwent the same procedure but were not subject to microwave energy.

Temperature measurements of organotypic raft cultures

A temperature probe (NOMAD-Touch, by Neoptix, Canada) was used to measure the temperatures of the rafts by placement on the surface of the tissues during the microwave treatment (Supplementary Figure S1).

Immunohistochemistry

Formalin fixed tissue cultures were paraffin-embedded and sectioned. Sections (2.5 μm) were stained with haematoxylin and eosin (H&E) or for antibody staining were subject to heat induced epitope retrieval (HIER). HIER methods were sodium citrate buffer pH 6 for all antibodies except p53 where treatment with EDTA buffer was carried out at pH 9. Sections were subsequently stained with the appropriate antibodies at the Veterinary Pathology Laboratory, University of Glasgow. Sections were imaged on an Olympus Bx57 microscope using either ×4, ×10 or ×20 lenses.

Immunofluorescence microscopy

3D tissues were fixed, embedded, sectioned and subjected to heat antigen retrieval prior to blocking in 10% donkey serum for 1 h at room temperature (RT). Slides were washed in phosphate buffered saline (PBS) and incubated in 5% donkey serum containing the primary antibody at 4 °C for 2 h or overnight. Slides were subsequently washed in PBS and incubated in 5% donkey serum containing the appropriate fluorescently labelled (AlexaFluor 488 or 555) secondary antibody (ThermoFisher Scientific) at RT for 1 h. Slides were washed in PBS and deionised water and coverslips mounted using ProLong Gold Antifade reagent with DAPI (ThermoFisher Scientific). Slides were visualised on a Zeiss LSM880 laser scanning microscope or on an LSM710 using ZEN 3.2 Blue software and fluorescence was quantified using ImageJ.

RNA extraction and cDNA synthesis

RNA was prepared from 3D tissues using QIAzol Lysis reagent and the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined using a Nanodrop One/One Microvolume UV–Vis Spectrophotometer (Thermofisher). cDNA was synthesised from total RNA (500 ng) using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR with DNase, according to the manufacturer's instructions.

Quantitative reverse transcriptase PCR (qRT-PCR)

Primers for the HPV16 bicistronic E6E7 transcript and the housekeeping gene β-actin, which was used as a reference, were designed using PrimerQuest (Integrated DNA Technologies). E6E7 Forward primer: 5′-CAATGTTTCAGGACCCACAG-3′, E6E7 reverse primer: 5′-CTGTTGCTTGCAGTACACACATTC-3′, E6E7 probe: 5′-CCACAGTTATGCACAGAGCTGC-3'. Beta-actin forward primer: 5′-AGCGCGGCTACAGCTTCA-3′, beta-actin reverse primer: 5′-CGTAGCACAGCTTCTCCTTAATGTC-3′, beta-actin probe: 5′-ATTTCCCGCTCGGCCGTGGT-3'. Quantitative Real-Time PCR was performed using a 7500 Real Time PCR System (Thermofisher). Each qRT-PCR reaction (total volume of 20 μl) included 10 μl of Takyon ROX Probe 2× MasterMix dTTP blue (Eurogentec), 4 μl of primer/probe mix (final concentrations of 900 nM and 100 nM for primers and probes respectively). 5 μl of cDNA was added. Reaction conditions were one cycle at 50 °C for 2 min, one cycle of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s followed by 60 °C for 1 min. Each sample was assayed in triplicate. Data produced in each qPCR reaction was analysed on the 7500 Real-Time SDS Software (Thermofisher). The threshold line for C_T determination was assigned automatically and was always within the exponential phase. Relative quantification of viral mRNA was done using the Livak method ($2^{-\Delta\Delta CT}$).

Statistical analysis

All experimental data shown are representative of at least three individual experiments. Statistical analysis

was performed with GraphPad Prism 7 using a student's T-test or for comparison between groups, an ANOVA Kruskal–Wallis test.

Role of the funding source

This work was funded by an Innovate UK Biomedical Catalyst grant (ID# 92138-556187), and by a Chief Scientist Office grant (TCS/19/11). We acknowledge support from the Medical Research Council (MC_UU_12014) as core funding for the MRC University of Glasgow Centre for Virus Research. None of the funding sources had any input into study design, the collection, analysis, and interpretation of data, the writing of the report or the decision to submit the paper for publication. All authors are employed by the University of Glasgow apart from A. Kirk who is a PhD student on an MRC-funded PhD programme at the University of Glasgow. Emblation Ltd provided the medical device but had no input into the study design, the collection, analysis, and interpretation of data, the writing of the report or the decision to submit the paper for publication.

Results

We adopted a three-dimensional (3D) epithelial raft culture system[24] to study the molecular impact of microwave treatment on cervical tumours *in vitro*. SiHa cells are derived from a grade II cervical squamous cell carcinoma.[21] They contain two integrated copies of the HPV16 genome per cell[30] and express HPV16 E6 and E7 oncoproteins.[31] Unlike other cervical cancer cell lines,[32] SiHa cells form undisrupted, non-invasive multilayered undifferentiated tissues in 3D cultures when grown at the air-liquid interface,[32] making them a useful model for this study (Fig. 1d).

Optimisation of microwave treatment

A range of treatment conditions were tested for temperature elevation in the SiHa 3D tissues due to microwave treatment: 5 W, 10 W or 15 W for time periods up to 40 s. Microwaves were applied to the SiHa tissues by inverting them onto the lid of a preheated plastic cell culture dish with the microwave device located on top of the tissue (Supplementary Figure S1). Triplicate temperature increase curves over a 10 s period are shown for 5 W (Fig. 1a) 10 W (Fig. 1b) or 15 W (Fig. 1c) treatments. Treatment with 10 W for 10 s resulted in the target temperature increase of between 45 and 48 °C (Fig. 1b). This thermal envelope and transient time is consistent with that of the tissues in plantar warts under an optimised protocol in use clinically, verified by in silico modelling using Multiphysics finite element analysis software (Comsol, Sweden) by the microwave device manufacturer. Compared to mock-treated SiHa tissue (Fig. 1d), treatment at 10 W for 10 s resulted in an immediate and discrete damage of the tissue at the treatment site (Fig. 1e). In the tissue areas proximal to the treatment site tissue integrity was compromised with gaps visible between cells, but away from the treated area the tissue appeared undisrupted. There was a clear demarcation between the treatment site and the untreated area, as observed *in vivo* in the plantar wart study.[18] We designated three tissues areas: "treated"–the area under the microwave probe, "proximal"–the adjacent area of disrupted tissue integrity and "distal"–undisrupted tissue (Fig. 1e). For a quantitative portioning of the treated tissue, we measured temperature change over 10 s at the centre ("treated" area) and distal areas of the tissues using two separate temperature probes (Supplementary Figure S1). A temperature increase of between 45 and 50 °C within 10 s was detected in the central portion of the tissue. In the distal portion, temperature only reached 32 °C after 10 s (Fig. 1f) demonstrating spatial precision of microwave energy treatment. The entire 3D tissue has a diameter of 14 mm. The 6.7 mm applicator head of the microwave device gave an average treatment area of 2.2 mm (±0.3 mm) diameter at the "treatment site", which was increased over time to around 3 mm diameter due to cell death (Fig. 1g). The disrupted "proximal" region measured an average width of 3.4 mm (±0.3 mm), which also increased over time to a maximum of 5.5 mm (Fig. 1h).

Fig. 2 shows haematoxylin and eosin (H&E)-stained images of treated, proximal and distal areas of tissues that were microwave-treated then re-incubated at 37 °C for up to 6 days to examine any tissue regrowth. This is the longest time period (20 days in total) tissue growth can be supported in 3D culture. Treated areas of the tissues did not regrow over this time (Fig. 2 compare image b and j). In the tissue areas proximal to treatment, growth was reduced at 72 h (Fig. 2g), but cells regrew after 144 h (Fig. 2k). In the distal areas, away from the treatment site (Fig. 2d, h, l) there was a reduction in tissue thickness at 72 h suggesting growth inhibition or ongoing cell death due to microwave treatment (Fig. 2m). However, tissue thickness recovered to a level very similar to 0 h treated tissue indicating that tissue growth was not permanently inhibited (Fig. 2m).

Microwave treatment reduces HPV-16 oncoprotein levels and re-introduces p53 and Rb expression

Cervical tumour progression is caused by increased expression of the HPV oncoproteins E6 and E7 and their repressive effects on p53 and Rb respectively. Therefore, next we measured levels of E6 and E7-encoding mRNAs. For this experiment, because we harvested RNA from the entire 3D tissue, we grew "mini" 3D tissues of 7 mm diameter to ensure that most cells in the tissue were exposed to microwave energy. Quantitative RT-PCR was carried out on cDNA synthesised from replicate RNA preparations from replicate tissues to detect E6/E7 bicistronic mRNA. No change was detected in E6/E7

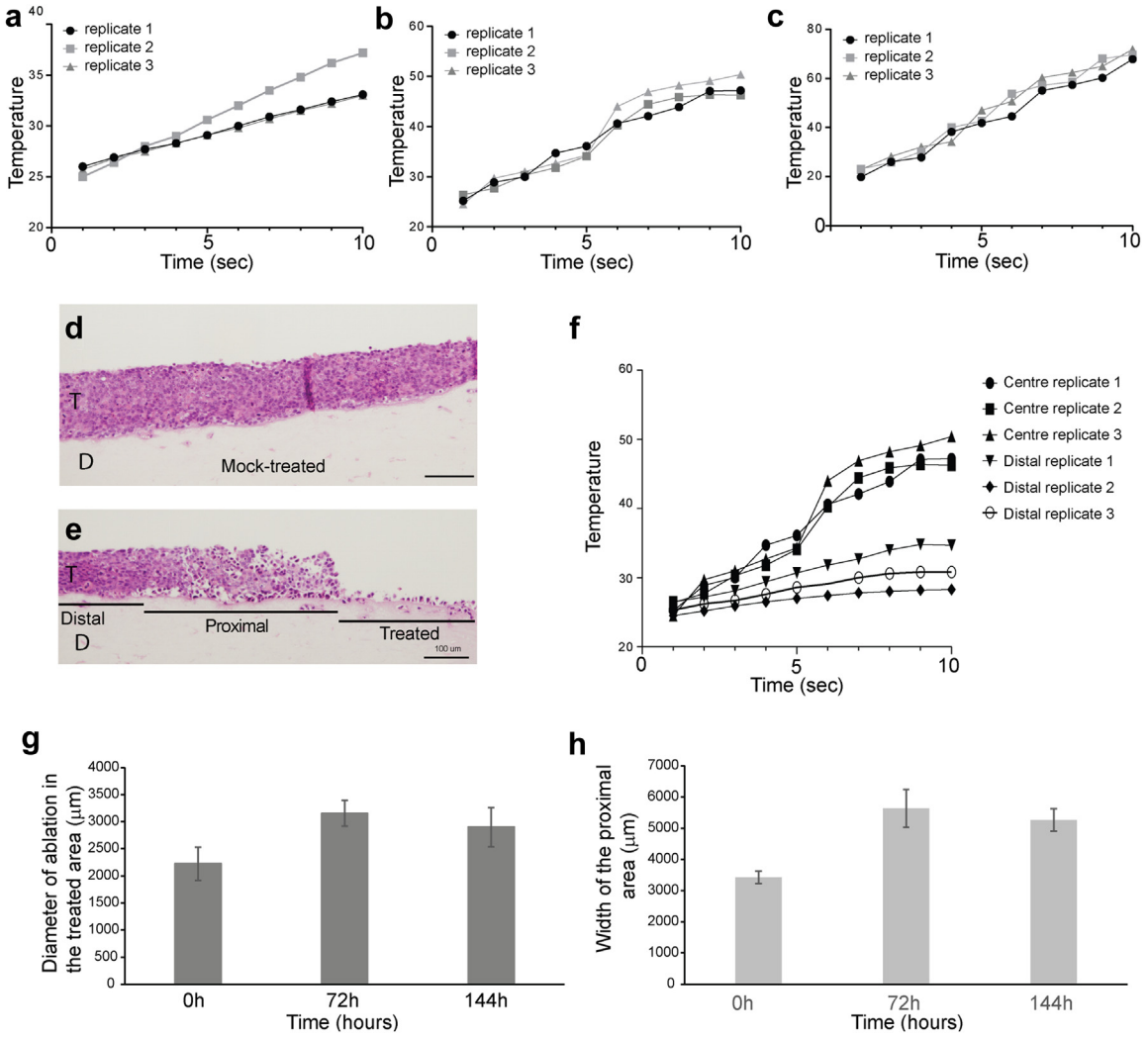

Fig. 1: **The effect of microwave treatment on three dimensional SiHa tissues.** Organotypic raft cultures of SiHa cells were grown at the air-liquid interface for 14 days then treated with microwaves at (a). 5 W, (b). 10 W, or (c). 15 W for up to 10 s. The graphs show the measurements of temperature increase every second over the 10 s time period in three independent tissues (replicates 1, 2 and 3) (d). H&E-stained section of a mock-treated formalin-fixed and paraffin-embedded (FFPE) SiHa tissue. (e). H&E stained FFPE section of a SiHa tissue treated with microwave energy at 10 W for 10 s. Both tissues were harvested immediately after treatment. The areas of the treated tissue we have designated as "treated" (cell disruption), "proximal" (loss of cell–cell contact) and "distal" (no significant tissue disruption) are indicated. T = SiHa tissue. D = collagen/fibroblast dermal equivalent. Scale bars in (d) and (e) =100 µM. (f). Graph of measurement of temperature increase every second over a 10 s period comparing temperature at the centre of the tissue (taken from Fig. 1(b)) with temperature at a distal portion of the same tissue in three replicate tissues (distal replicates 1, 2 and 3). A diagram of the experimental set-up is shown in Supplementary Figure S1(d). (g). Graph of the average diameter of the treated area of SiHa tissues over a 6-day period of re-incubation at 37 °C after microwave treatment. (e). Graph of the average diameter of the proximal area of SiHa tissues over a 6-day period of re-incubation at 37 °C after microwave treatment. Data in (g) and (h) show the average and standard error of the mean from five separate experiments.

mRNA expression in response to microwave treatment for the first 48 h following treatment (Fig. 3a). However, at 72 h there was a statistically significant decrease ($p < 0.05$) in E6/E7 mRNA expression. Next, we measured changes in E6 and E7 protein expression due to microwave energy. Following microwave treatment, 3D tissues were re-incubated at the air-liquid interface at 37 °C for 16 h prior to immunofluorescence staining with antibodies directed against E6 or E7. Fluorescence levels in 50 individual cells from treated, proximal and distal areas of three separate treated rafts were quantified (Fig. 3b and c). For both viral oncoproteins, a statistically significant reduction in fluorescence was detected in cells remaining in the treated area when

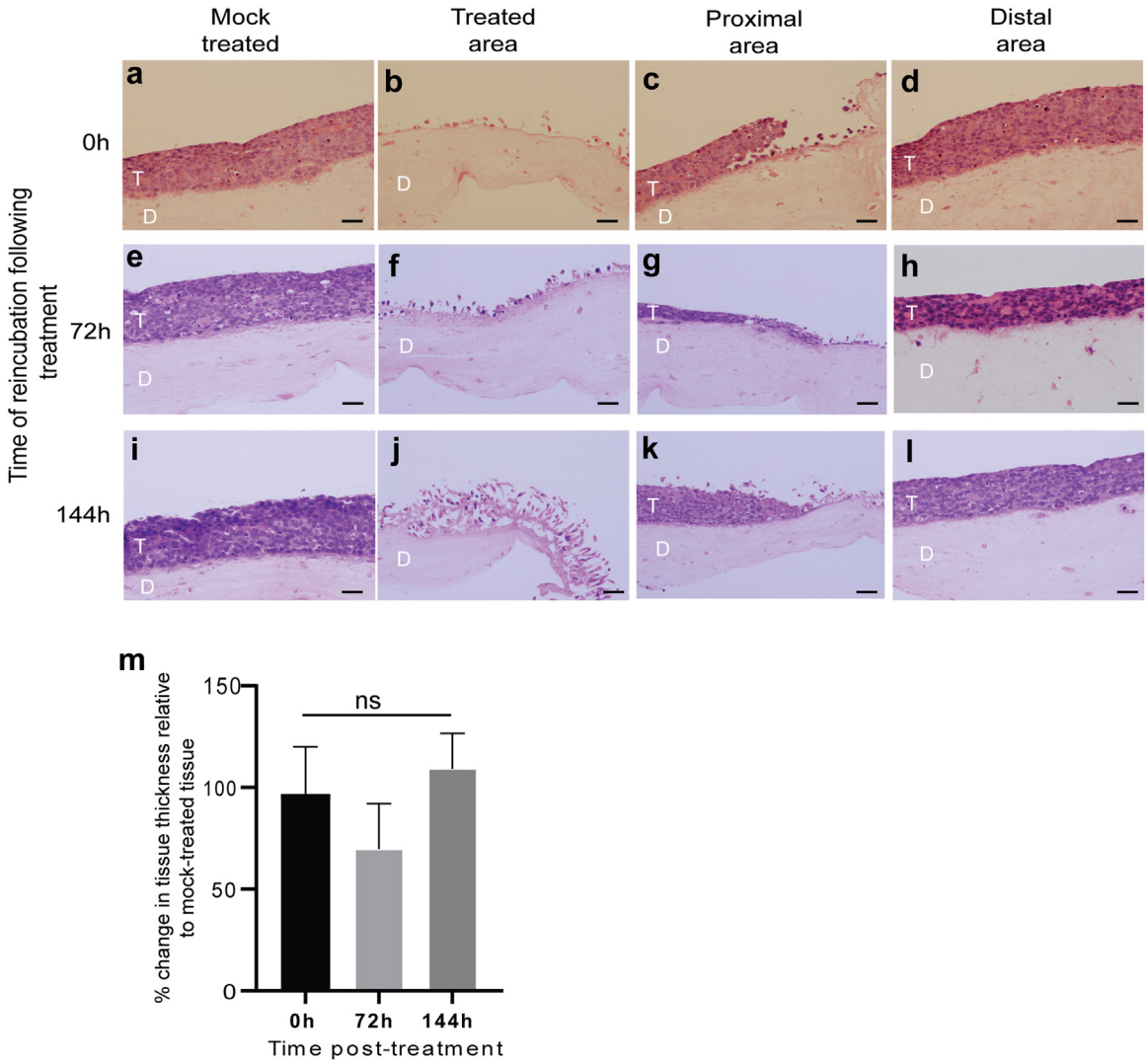

***Fig. 2:* Changes in growth of SiHa tissues due to microwave treatment over a 6-day period.** (a), (e), (i), mock-treated tissues re-incubated at 37 °C for 0, 72 or 144 h respectively following mock treatment. (b), (f), (j), images of the treated area of microwave-treated tissues re-incubated at 37 °C for 0, 72 or 144 h respectively following treatment. (c), (g), (k), images of the area proximal to the treated area of microwave-treated tissues re-incubated at 37 °C for 0, 72 or 144 h respectively following treatment. (d), (h), (l), images of the area distal to the proximal area of microwave-treated tissues re-incubated at 37 °C for 0, 72 or 144 h respectively following treatment. All images show H&E-stained 2.5 μm sections of FFPE *in vitro*-grown SiHa tissues. T = SiHa tissue. D = collagen/fibroblast dermal equivalent. Scale bars = 50 μM. The images are representative of five separate experiments. (m). Graph of changes in 3D tissue thickness at areas distal to the treated area at 0, 72 and 144 h post-microwave treatment. The data show the average and standard deviation from the mean from three separate experiments. ns = not significant $p > 0.05$ (student's t-test).

compared to distal areas of the tissues. Although less fluorescence was also detected in the area proximal to treatment, this was found to be statistically significant only for E7 protein.

Next, microwave-treated tissue sections were stained for the presence of the tumour suppressor and apoptosis activator, p53. E6 forms a complex with E6 associated protein (E6AP) and p53 to target p53 for proteasomal degradation.[33] Fig. 3d shows images of microwave-treated tissues (proximal area) which have been re-incubated at 37 °C for various times following treatment. A low level of p53 expression was detected at 0 h post treatment. At 2 h post-treatment increased p53 levels (26.7-fold increase) were apparent in the upper layers of the tissue and p53 levels increased (74.0-fold increase) up to 16 hrs post treatment. At this time, almost all the cells appeared positive for p53. High risk HPV E7 protein degrades the cell cycle check point

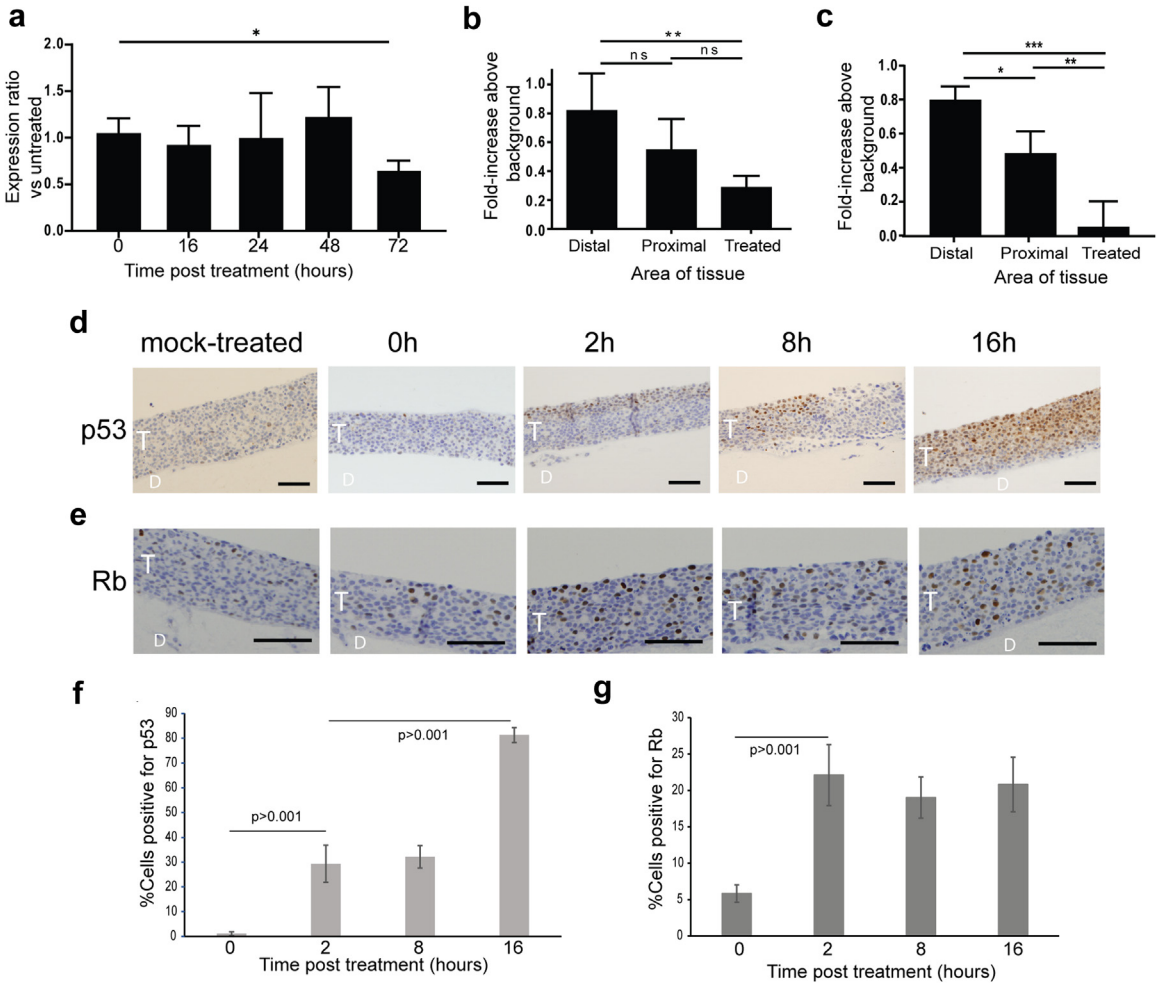

***Fig. 3:* Microwave treatment reduces HPV oncoprotein levels and re-introduces p53 and Rb expression.** (a). "Mini" 3D SiHa tissues of 7 mm diameter were microwave-treated with 10 W for 10 s and either fixed immediately (0hr) or re-incubated at the air-liquid interface at 37 °C for 16, 24, 48 or 72 h prior to harvesting for RNA extraction. Following cDNA synthesis, levels of E6/E7 bicistronic mRNA were quantified by RT-qPCR. Data are expressed as changes in E6/E7 mRNA levels relative to changes in beta-actin mRNA levels (ΔΔCt) and relative to levels in mock-treated tissues. The data shown are the mean and standard deviation from the mean of three separate experiments using three independent tissues. *p < 0.05 (student's t-test). Sections of microwave-treated tissues were immunofluorescence stained with an antibody against (b) HPV16 E6, or an antibody against (c) HPV16 E7. Immunofluorescence intensity was quantified using ImageJ in 50 individual cells in the treated, proximal and distal areas of three separate 3D SiHa tissues. (b). Graph of E6 protein levels in the distal, proximal and treated areas of SiHa tissues that were microwave-treated then reincubated for 16 h. (c). Graph of E7 protein levels in the distal, proximal and treated areas of SiHa tissues that were microwave-treated then reincubated for 16 h. The graphs show the mean and standard deviation from the mean using data from three separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (student's t-test). (d). Immunohistochemistry staining with an antibody against p53 of SiHa tissues mock-treated or microwave-treated and reincubated for 0, 2, 8 or 16 h. (e). Immunohistochemistry staining with an antibody against Rb of SiHa tissues mock-treated or microwave-treated and reincubated for 0, 2, 8 or 16 h. Cell nuclei are counterstained with haematoxylin (blue stain). T = SiHa tissue. D = collagen/fibroblast dermal equivalent. Data shown are representative of three individual experiments. Scale bars = 50 μm. (f). Graph showing quantification of percentage cells positive for p53 protein in tissues at 0, 2, 8, and 16 h following microwave treatment. Percentage positively stained cells in three replicate tissues were quantified by ImageJ. At least 150 cells in each of five replicate tissues were counted for each time group. p-values were determined by student's t-tests. (g). Graph showing quantification of percentage cells positive for Rb protein in tissues at 0, 2, 8, and 16 h following microwave treatment. Percentage positively stained cells in three replicate tissues were quantified by ImageJ. At least 150 cells in each of 6 replicate tissues were counted for each time group. Graphs show the average and standard deviation from the mean. p-values were determined by student's t-tests.

inhibitor Rb.[26] Due to reduced E7 levels, increased Rb levels (3.8-fold increase) were detected within 2 h post-microwave treatment and this was sustained over a 16 h period (Fig. 3e). Quantification showed that the observed increases in p53 and Rb levels were statistically significant (Fig. 3f and g). Taken together, these findings suggest that microwave treatment reduces the expression of HPV E6 and E7 oncoproteins, concomitant with an increase in expression of p53 and Rb.

Microwave treatment reduces cellular proliferation and induces apoptosis

The observed increase in p53 and Rb levels would allow for apoptosis to resume while inhibiting cell proliferation. Next, we used immunohistochemistry staining to test this directly.

Mock microwave treatment was compared to treatment at 10 W for 10 s, followed by re-incubation at 37 °C for 16 h. Cleaved caspase 3 staining of treated tissue sections revealed induction of apoptosis only in microwave-treated tissues (Fig. 4a). Quantification of caspase 3 levels showed significantly increased expression in treated tissues compared to mock-treated tissues (Fig. 4b). The opposite effect was seen in tissues stained for a marker of cellular proliferation, MCM2 (mini chromosome maintenance protein 2) (Fig. 4c), with significantly fewer positive cells being detected in the microwave-treated tissue (Fig. 4d). Increased cleaved caspase 3 expression in microwave-treated tissues was sustained over 72 h, especially in the proximal tissue regions (Fig. 3e). Ki67, a second marker of cell proliferation, was repressed up to 24 h post-treatment in both proximal and distal areas of treated tissues (Fig. 3f). Some increase in cell proliferation was observed in the distal areas between 48- and 72-h following treatment compared to time periods up to 24-h post-treatment. Our results show that treatment with microwave energy resulted in induction of apoptosis as well as an inhibition of cellular proliferation i.e., a reversal of the tumour phenotype.

Microwave treatment induces a heat shock response

Heating to fever temperature (>38 °C) and above induces thermal stress, which can be reversible or can result in cell death. Microwave therapy has previously been shown to induce heat shock protein expression in skeletal muscle.[34] To determine if a heat shock response was induced in the 3D tissues following microwave treatment, tissues (treated and re-incubated for 16 h) were immunohistochemically stained for the molecular chaperone HSP70, whose expression is known to create a thermotolerant cellular environment.[35] An entire HSP70-stained tissue section is shown in Fig. 5a. Due to the cutting of the section away from the site of direct microwave treatment site, no area devoid of cells is visible in this tissue section. Thus, the tissue section represents the proximal and distal areas of the tissue. The lower panels at ×20 magnification (Fig. 5b) clearly show HSP70 induction in areas proximal to the microwave site due to direct microwave effect. Only low levels of HSP70 staining were detected in areas distal to the treatment site at the ends of the tissue (Fig. 5b). Examination of treated areas of the tissues revealed HSP70 expression in cells remaining in the treated area and in the area proximal to treatment (Fig. 5c). These data show that microwave treatment induces a localised heat shock response.

Microwave treatment induces a cellular stress response

Cell stress leads to translational stress. HSP70 family members alter mRNA metabolism, including mRNA decay and translation.[36] During stress, untranslated or translation-stalled cytoplasmic mRNAs are sequestered into stress granules.[37] To investigate whether a stress response was induced by microwave energy, induction of G3BP a marker of cellular stress granule formation, was examined by immunofluorescence microscopy. In the absence of cellular stress, G3BP staining appears diffuse, however, under conditions of prolonged stress the staining becomes punctate. A mock-treated tissue was included in the analysis, as well as a positive control tissue that had been incubated at 40 °C in culture for 6 h to induce heat shock (Fig. 5d + ve control). The mock-treated tissue showed no G3BP staining (Fig. 5d Mock-treated) while G3BP was clearly induced due to heating in the positive control tissue (Fig. 5d + ve control). In microwave-treated tissues, distal regions showed low levels of G3BP staining (Fig. 5d "Distal") but there was an increase in G3BP in the proximal and treated regions. Punctate staining consistent with cytoplasmic granules was visible in the cells at the edge of the proximal region (Fig. 5d, "Proximal", white arrowheads) and in cells in the in the treatment site (Fig. 5d "Treated", white arrowheads) indicating stress granule formation. Another stress granule protein, PABPC1 was examined in SiHa tissues treated with microwaves. G3BP and PABPC1 levels were both increased in the 16-h re-incubated tissues compared to 0 h post-treatment (Fig. 5e). There was clear cytoplasmic co-localisation of G3BP and PABPC1 at 0 h which was increased at 16 h post-microwave treatment (Fig. 5e). These data reveal that microwave treatment produces a robust heat shock response leading to translational stress. Since microwave treatment does not significantly inhibit HPV oncogene transcription at 16 h post treatment yet viral oncoprotein expression is reduced, our data suggest that the stress response due to microwave energy inhibits translation of HPV oncoproteins E6 and E7.

Microwave treatment effects on in vitro-grown HPV16-infected precancer tissues

Microwave therapy may prove most useful to treat HPV-positive precancers to inhibit cancer progression.

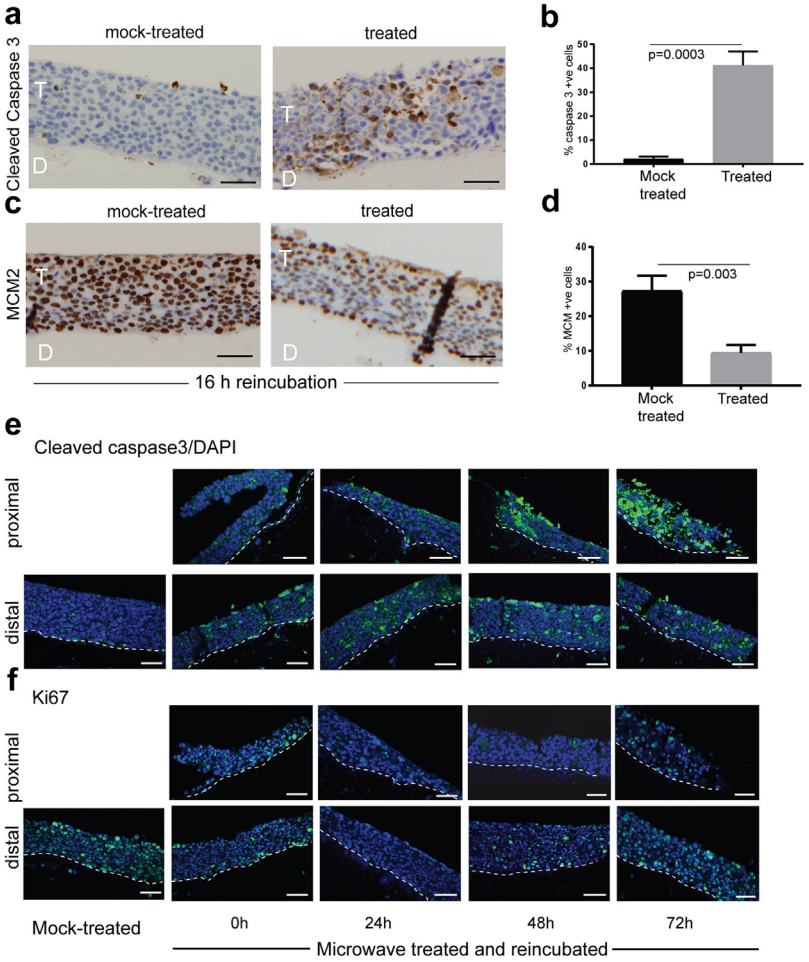

Fig. 4: **Microwave treatment induces apoptosis and reduces cellular proliferation.** SiHa tissues were mock-treated or microwave-treated with 10 W for 10 s then re-incubated at 37 °C for 16 h. (a) 2.5 µm sections of FPPE tissues were immunohistochemistry stained with an antibody against cleaved caspase 3 (apoptosis marker). Brown staining indicates the presence of the protein of interest. Nuclei are stained blue by haematoxylin. T = SiHa tissue. D = collagen/fibroblast dermal equivalent. Scale bars = 50 µm. (b). Graph of gain of cleaved caspase 3 staining in microwave-treated tissues compared to mock-treated tissues. Percentage positively stained cells in three replicate tissues was quantified by counting stained nuclei in at least 150 cells in three replicate tissues. The graph shows the average and standard deviation from the mean. p-value was determined by student's t-test. (c) 2.5 µm sections of FPPE tissues were immunohistochemistry stained with an antibody against MCM2 (cellular proliferation marker). Brown staining indicates the presence of the protein of interest. Nuclei are stained blue by haematoxylin. The thick black lines in the treated tissue are caused by tissue fold-over at these points. T = SiHa tissue. D = collagen/fibroblast dermal equivalent. Scale bars = 50 µm. (d). Graph of loss of MCM2 staining in microwave treated tissues compared to mock-treated tissues. Percentage positively stained cells in three replicate tissues were quantified by counting stained nuclei in at least 150 cells in three replicate tissues. The graph shows the average and standard deviation from the mean. p-value was determined by student's t-test. (e). Cleaved caspase 3 immunofluorescence staining (green staining) of mock-treated or microwave-treated tissues re-incubated at 37 °C for the indicated times following treatment. (f). Ki67 (cellular proliferation marker) immunofluorescence staining (green staining) of mock-treated or microwave-treated tissues re-incubated at 37 °C for the indicated times following treatment. Nuclei are counter stained with DAPI (blue). White dotted lines show the basal layer of the tissues and junction with the dermal equivalent. Scale bars = 50 µm. Data shown are representative images from three separate experiments.

Therefore, next we tested the effect of microwave energy on 3D cultures of NIKS16 cells, a model of HPV16-positive anogenital intraepithelial neoplasia.[23] All the images in Fig. 6 are of distal areas of microwave-treated tissues but the proximal and treated areas of replicate tissues showed similar cell proliferation and differentiation changes. Microwave treatment resulted in marked structural changes to the tissue with increased keratinisation at 48 h post-treatment (Fig. 6a). A reduction in expression of MCM2 and Ki67 over 48 h indicated decreased cell proliferation (Fig. 6b and c). This was accompanied by an increase in expression of differentiation markers keratin 10 and involucrin (Fig. 6b and c). These data reveal a rebalancing from proliferation to

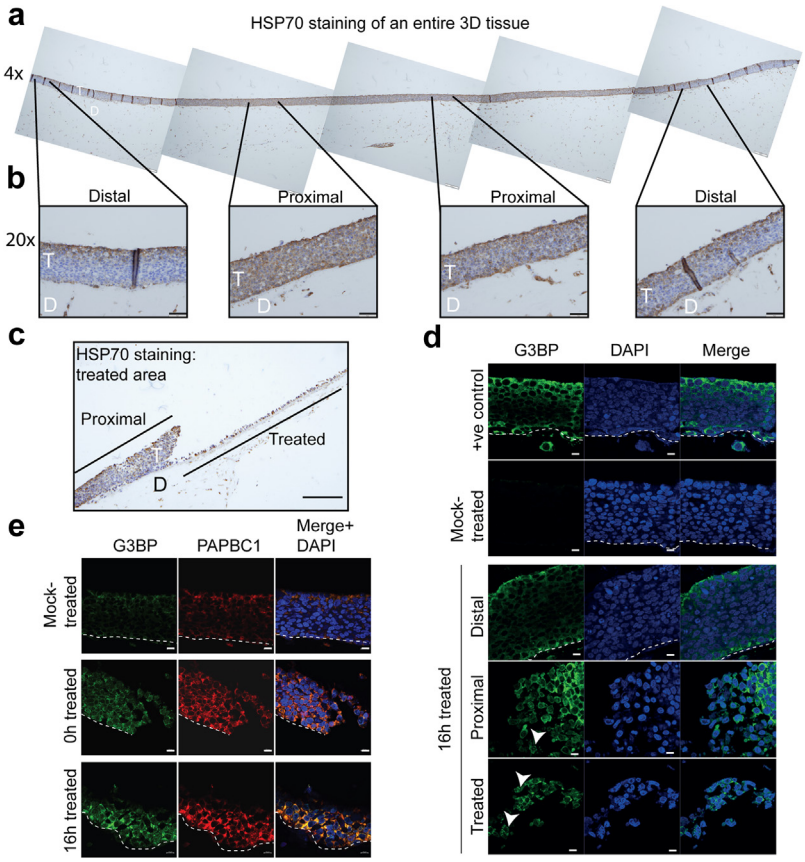

***Fig. 5:* Microwave treatment induces a cellular stress response.** (a). 4× magnified image of an entire 3D SiHa tissue microwave-treated and re-incubated at 37 °C for 16 h. Immunohistochemistry staining for HSP70 expression is shown. (b). 20× magnified panels of two distal and two proximal regions from the tissue in (a). T = SiHa tissue. D = collagen/fibroblast dermal equivalent. No "treated" region is visible because the 3D tissue has been sectioned behind the zone of tissue disruption. Scale bars = 50 μM. (c). Immunohistochemistry staining for HSP70 of a tissue microwave-treated and re-incubated at 37 °C for 16 h showing the treated and the proximal areas. T = SiHa tissue. D = collagen/fibroblast dermal equivalent. Scale bar = 50 μM. (d) Immunofluorescence staining for G3BP (green staining) was performed on heat-shocked (+ve control: incubated at 40 °C in culture for 6 h), mock-treated, and microwave-treated SiHa tissues which had been re-incubated at 37 °C for 16 h. Staining in distal, proximal and treated areas of a treated tissue is shown. Arrowheads indicate G3BP-positive cytoplasmic granules. (e). Immunofluorescence co-staining with antibodies against G3BP (green staining) and PABPC1 (red staining) of mock-treated or microwave-treated SiHa tissues fixed immediately following treatment (0 h) or re-incubated at 37 °C for 16 h. Blue staining = DAPI. White dotted lines show the basal layer of the tissues and junction with the dermal equivalent. Scale bars = 10 μm. Data shown are representative images from three separate experiments.

differentiation due to microwave treatment. A similar effect was seen in HPV18-positive 3D tissue cultures (Supplementary Figure S2). Expression levels of E6 and E7 oncoproteins are too low to detect efficiently in NIKS16 cells by antibodies. Therefore, we were unable to measure loss of viral oncoprotein expression in these tissues. However, the increased differentiation noted in microwave-treated tissues suggests an abrogation of E6 and E7 expression since these proteins inhibit differentiation in infected tissues.[29,30]

Like SiHa tissues, microwave treatment of NIKS16 tissues induced sustained expression of cleaved caspase 3, and the autophagy markers LC3B and p62 (Fig. 7a). Finally, we confirmed that microwave treatment induced HSP70 (Fig. 7b). Induction of HSP70 expression was rapid, within the first 4 h of treatment, and was sustained for 16 h post-treatment (Fig. 7c). This resulted in translation stress in the NIKS16 tissues because levels of the stress granule marker G3BP were increased in the cytoplasm of treated cells (Fig. 7d). These data confirm the cellular response to microwave treatment in a different type of HPV-infected tissue.

Discussion

The microwave treatment protocol that we developed resulted in precise tissue disruption at the target site. The dimensions of the area of treatment in SiHa cells

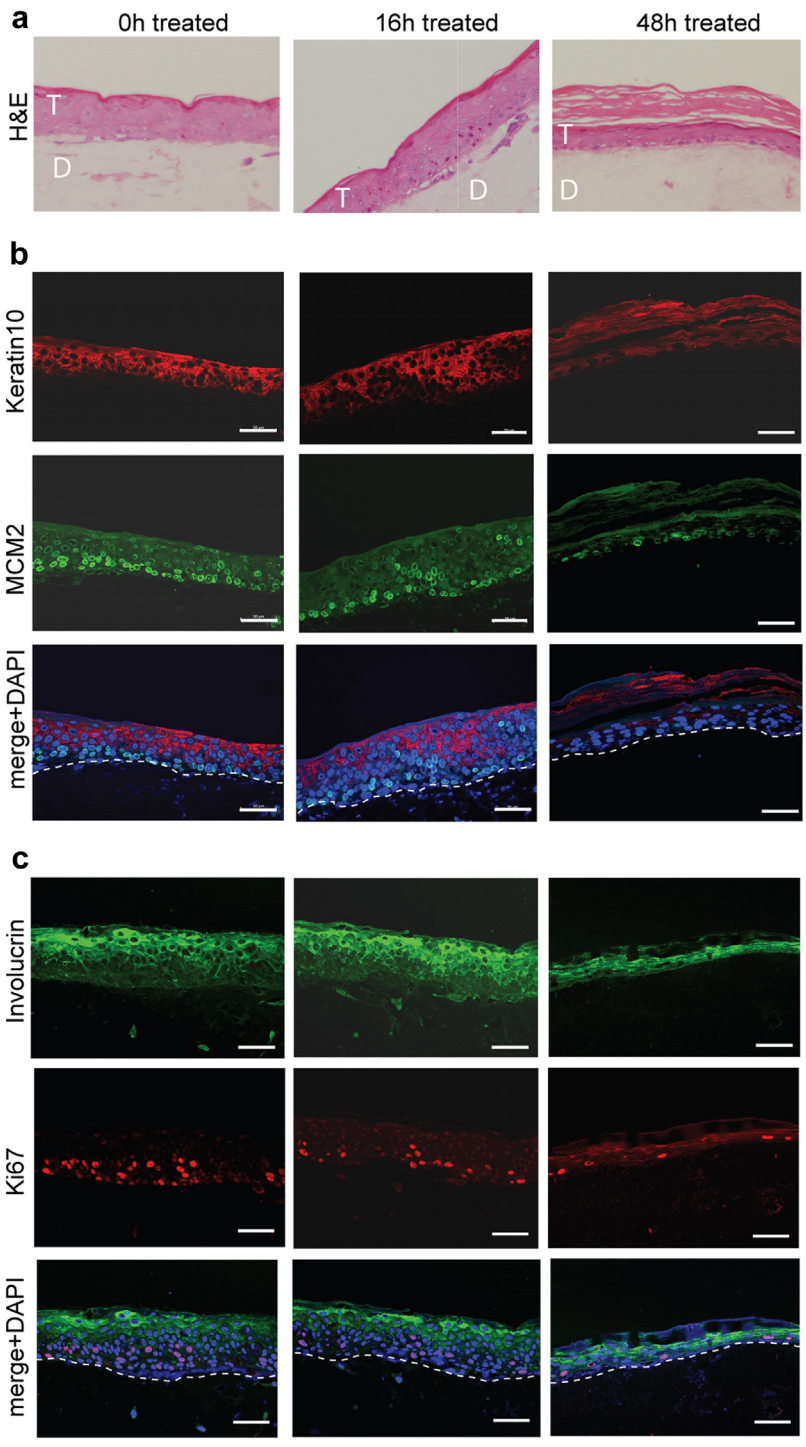

Fig. 6: **Effect of microwave treatment on in vitro-grown 3D NIKS16 tissue**s. (a). H&E-stained sections of NIKS16 tissues microwave-treated and reincubated for 0, 16 and 48 h. T = SiHa tissue. D = collagen/fibroblast dermal equivalent. (b). Immunofluorescence staining of distal areas of microwave-treated NIKS16 tissues (microwave-treated and reincubated for 0, 16 and 48 h) with antibodies against epithelial differentiation marker keratin 10 (red staining) and cell proliferation marker MCM2 (Green staining). The bottom image in each column shows the merged images and nuclei stained with DAPI. (c). Immunofluorescence staining of distal areas of microwave-treated NIKS16 tissues (microwave-treated

agreed well with those measured *in vivo* in the previous plantar wart study and there was no significant spreading into the dermal equivalent layer as expected due to the limited depth of penetration of microwave energy.[18] Cellular apoptosis was detected in areas proximal to the treatment site. Induction of apoptosis in the tissues is in agreement with another study where microwave treatment was shown to induce caspase-dependent apoptosis in a leukaemic cell line.[38] Apoptosis was sustained over at least 72 h and was also observed at sites distal to the treatment site, but at a lower level than that observed at proximal sites. Autophagy markers LC3B and p62 were upregulated upon heat shock in both SiHa (data not shown) and NIKS16 tissues. Following re-incubation at 37 °C after microwave treatment, cells in the area outside of the treatment site in SiHa tissues continued to grow over a 6-day period but there was evidence of reduced proliferation as shown by reduced expression of Ki67 compared to mock-treated tissues. Importantly, no regrowth in the treated area was observed. Taken together, the data suggest that microwave energy could be used to precisely treat anogenital lesions, kill HPV-infected tumour cells and inhibit regrowth from the surrounding area.

The heat shock response is a normal response of cells to ensure survival under conditions of environmental stress. As expected, microwave treatment induced a localised heat shock response due to hyperthermia.[34] Of all the heat shock chaperone proteins, HSP70 is the most strongly induced by cell stress and it acts to modulate the structure of misfolded proteins that accumulate as a direct result of cell stress.[35] In microwave-treated tissues, HSP70 was specifically and rapidly induced in and around the treatment site. This emphasises the locus-restricted nature of microwave-induced hyperthermia. The heat shock response also induces formation of stress granules which contain mRNA/protein assemblies from stalled translation initiation events.[37] Following adverse cellular events, stress granules can be remodelled to allow re-initiation of protein translation or to allow autophagy.[36] Analysis of G3BP and PABPC1, revealed stress granule formation particularly in cells of the treated and proximal tissue areas following microwave treatment. Therefore, we suggest that microwave hyperthermia leads to heat shock and translational stress followed by cell death at the treatment site, but the cell stress response is muted at sites distal to the treated area. While we found that E6E7 bicistronic mRNA expression was unaltered upon microwave treatment, except at later time points, there was a statistically significant decrease in E6 and E7 protein expression. This result implies that heat regulation of E6 and E7 expression occurs at a post-transcriptional level, possibly at the level of translational arrest and sequestration of the E6/E7 mRNAs into stress granules.[37]

In HPV-positive cancer cells such as SiHa cells, E6 forms a complex with p53 and E6AP leading to p53 proteasomal degradation.[25,33] Cellular heat shock increases expression of p53 and leads to its phosphorylation and subsequent tetramer formation in the nucleus. This results in stabilisation of p53[39–42] which can then activate transcription of HSP70, leading to the heat shock response and apoptosis.[43] Our data suggest a heat-shock-induced reduction in E6 protein levels, which should add to the increased p53 levels due to hyperthermia. Taken together, these dual control mechanisms explain the rapid and significant p53 upregulation detected upon microwave treatment at the treatment site, and in areas proximal to the treatment site. A similar result in conventional cell culture of SiHa cells was reported previously where hyperthermia caused loss of E6, preventing p53 degradation. In that study, de novo synthesis of p53 was also detected and this led to the normal induction of apoptosis in HPV-positive cancer cells.[44] A therapy which induces p53 and apoptosis of cervical cancer cells shows promise for anticancer therapy.

As well as influencing the E6/p53 axis, microwave treatment resulted in upregulated levels of the E7 target protein Rb. Normally Rb controls the transition from G1 to S phase of the cell cycle. Phosphorylation of Rb by cyclin dependent kinases allows release of E2F transcription factor to promote transcription of cell cycle-related genes. HPV E7 binds Rb and releases E2F to activate expression of cell cycle-related genes.[26] Thus, loss of E7 due to hyperthermia should repress cell proliferation and inhibit G1 to S-phase cell cycle progression. Our observation of decreased levels of S-phase-specific proteins MCM2 and Ki67 upon microwave treatment supports this conclusion. However, under conditions of cell stress, p38 stress-activated protein kinase represses E2F through selective phosphorylation of Rb and disallowing its cyclin-dependent kinase phosphorylation. p38-phosphorylated Rb now has increased affinity for E2F. This results in downregulation of transcription of E2F-regulated cell cycle-related genes and inhibition of cell-cycle progression.[45] Therefore, although E7 levels may be repressed in microwave-treated cells, Rb can no longer activates cell proliferation due to the cell stress response.

and reincubated for 0, 16 and 48 h) with antibodies against epithelial differentiation marker involucrin (green staining) and cell proliferation marker Ki67 (red staining). The bottom image in each column shows the merged images and nuclei stained with DAPI. White dotted lines show the basal layer of the tissues and junction with the dermal equivalent. Scale bars = 50 μM. Data shown are representative images from three separate experiments.

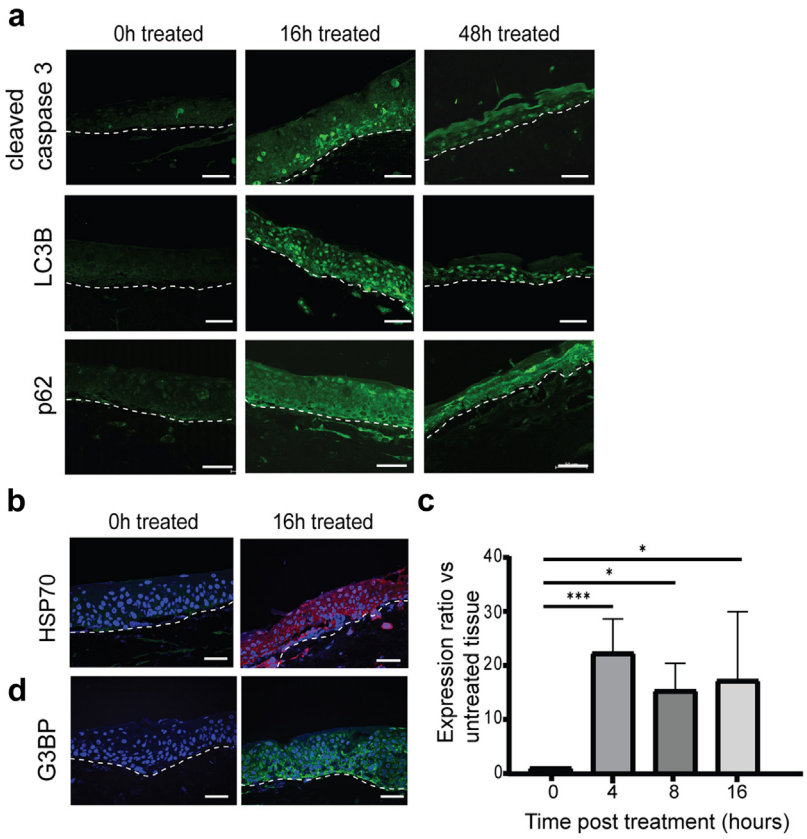

***Fig. 7:* Microwave treatment results in apoptosis and autophagy and induction of a heat shock response.** (a). Immunofluorescence staining (green staining) of microwave-treated NIKS16 tissues (microwave-treated and reincubated for 0, 16 and 48 h) with antibodies against cleaved caspase 3, LC3B and p62. (b). Immunofluorescence staining of microwave-treated NIKS16 tissues (microwave-treated and reincubated for 0 and 16 h) with an antibody against HSP70 (red staining). (c). Increase in HSP70 mRNA expression in NIKS16 3D tissues over time following microwave treatment and reincubation. ***$p < 0.001$. *$p < 0.05$. p-values were determined using student's t-tests. (d). Immunofluorescence staining of microwave-treated NIKS16 tissues (microwave-treated and reincubated for 0 and 16 h) with an antibody against and G3BP (green staining). Nuclei are counterstained with DAPI. White dotted lines show the basal layer of the tissues and junction with the dermal equivalent. Scale bars = 50 μM. Data shown are representative images from three separate experiments.

In summary, the effects of E6 and E7 reduction in microwave-treated cells may be enhanced by the manner in which p53 and Rb respond to cell stress. Since HPV-associated cancer progression is initiated and sustained by increased expression of the viral oncoproteins E6 and E7, our data suggest that microwave treatment could reverse the tumour phenotype of cervical cancer cells.

Hyperthermia can cause HSP70 to be released into the environment.[46] HSP70 is a DAMP which can activate antiviral and antitumoural pathways.[10] Cell-released HSP70 can bind antigen presenting cells,[47,48] but as a chaperone, HSP70 can be released bound to tumour cell antigens. This results in uptake and presentation of the antigens by antigen presentation cells leading to induction of anti-tumour $CD8^+$ T-cell responses.[49] Therefore, microwave treatment has potential to stimulate cell-mediated immunity. This is particularly relevant in the case of treatment of HPV-associated lesions since stimulation of the immune response could clear the virus and prevent reinfections. In the plantar wart study, microwaves potentiated cutaneous immunity to HPV[18] while in studies of genital warts, hyperthermia upregulated APOBEC antiviral activity[50] and induced a range of proteins involved in antiviral responses.[51] Immune activation could result in clearance of lesions not only at the treatment site but at other, untreated sites as suggested in a study of hyperthermia to treat genital warts.[50] This possibility requires further investigation of microwave therapy against HPV-associated cancers and precancers *in vivo*.

Compared to ablative procedures such as cryo- or laser therapy, microwave therapy can have a greater depth of penetration. Moreover, it produces no vapour or smoke[52] and so is safe for resolution of virus-positive lesions. The small size of the treated area is also

advantageous and different device probes can be manufactured to fit particular clinical uses. In the *in vivo* verruca and actinic keratoses studies, participants experienced pain from microwave treatment but it was bearable and transient.[18,19] These studies support favourable therapeutic effects *in vivo*, particularly for the localised nature of the treatment. Although treatment of mucosal tissue *in vivo*, as opposed to cutaneous sites could result in a higher level of pain, this issue could be solved using local anaesthesia. If microwave treatment induced antiviral or antitumoural immunity, local recurrence could be alleviated while lesions at untreated sites might regress.

The most likely clinical use for a microwave device is in treatment of genital warts and anal and vulvar precancers and cancers, especially where multifocal disease occurs. The microwave device is currently CE marked for dermatology use and so cannot currently be used to examine efficacy in the clinic in treating these diseases. Although it has allowed us to understand the molecular basis of microwave-induced tissue apoptosis, the *in vitro* nature of our study is a major limitation. The nature of microwave heating in various tissues is known to be different such as liver, warts or actinic keratosis[15,18,19] where the power and time are adjusted to suit the desired therapeutic envelope. These adjustments can be model led in computer simulation prior to conducting treatment of any new site. However, any *in vivo* study on anogenital lesions would require careful assessment of treatment regimes, treatment tolerance and efficacy for each type of lesion. It will be very important to monitor any disease recurrence over time, a factor that has not yet been analysed in the published *in vivo* studies.

In conclusion, our data show that inhibition of cell proliferation and induction of apoptosis in HPV-positive cervical tumour tissues can be induced by hyperthermia delivered in a precise, highly localised manner by microwaves. The mechanism of cell death is through activation of the cellular stress response and repression of HPV oncoprotein expression. Precision microwave delivery may present a potential new treatment for HPV-positive anogenital precancerous lesions and cancers.

Contributors

All authors have read and approved the final version of the manuscript.

MC: investigation, data interpretation, drafting the manuscript. Verified the underlying data.

IE: investigation, data analysis. Verified the underlying data.

AK: investigation, data analysis. Verified the underlying data.

AS: investigation, data analysis, project administration.

SVG: conceptualisation, funding acquisition, supervision, visualisation, writing the manuscript. Verified the underlying data.

Data sharing statement

All data generated or analysed during this study are included in this report, and are available upon request from the corresponding author. All materials are commercially available.

Declaration of interests

Emblation Ltd (Alloa, Scotland) provided the microwave device. Emblation Ltd had no input into experimental design, data collection or analysis. We declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104577.

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ORIGINAL ARTICLE

The treatment of plantar warts using microwave—A review of 85 consecutive cases in the United States

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Abstract

Background: Plantar warts (verrucae plantaris) are a common source of pain for patients and are often refractory to treatment. Previous work has shown a high clearance rate of verrucae using a surface-based microwave device (Swift®).

Aims: To assess the efficacy, defined as the complete visible clearance of warts, in patients with verrucae plantaris receiving microwave treatment.

Patients: We undertook a retrospective review and identified records of 85 patients who underwent a course of microwave treatment at a single US-based podiatry centre. Efficacy was analyzed on the basis on intention-to-treat.

Results: In patients who received ≥1 session there was a complete clearance rate of 60.0% (51/85) (intention-to-treat; 59 patients completed treatment, 26 lost to follow-up) and 86.4% (51/59) per treatment completion; no significant differences in clearance rates of children and adults were observed (61.0% [25/41] vs. 59.1% [26/44]). There were 31 patients who received three sessions of microwave therapy with a clearance rate of 71.0% (22/31) as per intention-to-treat (27 patients completed treatment, 4 lost to follow-up). An average of 2.3 sessions (SD: 1.1; range: 1–6) was required for the complete clearance of plantar warts. Complete clearance was also observed in some patients with recalcitrant warts following additional treatment sessions (42.9% [3/7]). A significant reduction in wart related pain was reported for all patients undergoing treatment. Some patients continued to report a reduced amount of pain post-therapy compared with pretherapy.

Conclusions: Microwave treatment of verrucae plantaris appears to be a safe and effective procedure.

KEYWORDS
foot, HPV, microwave, verruca, wart

1 | INTRODUCTION

Warts are a common dermatological diagnosis caused by the human papillomavirus (HPV). Lesions arising on the skin can present a therapeutic challenge, particularly those arising on the plantar surface of the foot (verrucae plantaris). Clinical experience and guidelines suggest that plantar lesions are more resistant to traditional treatments like cryotherapy and salicylic acid compared with lesions elsewhere on the skin leading to chronicity, particularly in adults.[1] Moreover, lesions can be a cause of embarrassment and limit sporting and other activities.[2] In 2000, Dyall-Smith remarked how little verruca treatments had changed since the 1950's and this still holds true today.[3,4]

Traditionally, freezing of warts or cryotherapy has been the mainstay of treatment. However, research in the last decade or so has studied the positive effects of heating the skin in the treatment of tumors and skin cancers.[5] Heating the skin to just above normal body temperature (hyperthermia) using various methodologies has been shown to have a range of positive benefits on modifying the immune response.[6] The Swift® microwave unit (Emblation Limited, UK) is a medical device licensed in the USA and Canada for the treatment of surface lesions by utilizing microwave energy to heat skin through the application of a probe.[7,8] The device was FDA cleared in 2018 for the coagulation of soft tissue during noninvasive procedures.[7] It was Health Canada approved in 2017 for microwave treatments in the fields of dermatology and podiatry.[8] To date, there has been no efficacy data reported from the United States for the treatment of plantar warts. The present study reviews the outcomes of microwave treatment for plantar warts from an early adopting podiatry clinic in New York.

2 | METHODS

We performed a retrospective review of all patients who had undergone a course of microwave treatment for their verrucae plantaris at one podiatry practice location. All patients provided consent. Exclusion criteria were diabetics, those with peripheral vascular disease as evidenced by lack of palpable pedal pulses, and women who were pregnant. Pain symptoms associated with warts were assessed using a 10-point pain scale (ranging from 0—no pain to 10—worst pain imaginable) prior to the first treatment and at the final assessment appointment.

A course of treatment was defined as at least one session of microwave treatment with a follow-up appointment at least 4 weeks after the last session. Treatment response was defined as complete visible clearance of the wart, with return of the normal dermatoglyphics across the skin previously occupied by the wart. In each treatment session, local hyperthermia was induced through repeated microwave applications lasting 2 s each, which supplied energy at a frequency of 8 GHz and an average power of 8.0 W (SD: 0.3; range 6–8). Pretreatment paring or debridement were not routinely performed unless the wart surface did not provide a flat surface for the microwave applicator tip. Each wart received an average of five microwave applications per session (SD: 0.1; range 5–6). After the primary session of treatment, additional treatment sessions could be provided at a follow-up if treatment response was not achieved. If treatment discomfort occurred, administration of a local or topical anesthetic was considered.[9] All verrucae were treated and reviewed at each visit by the same clinician (RMC).

Of note, patient treatment was ongoing during the period of the US SARs-CoV2 (COVID-19) pandemic. Review of data records in this period resulted in an undesirable proportion of subjects (40%) not pursuing full courses of treatment and follow-up as a result of ongoing pandemic restrictions of travel and lifestyle. To account for the missing data in as conservative a method as possible, an intention-to-treat (ITT) analysis was primarily used where subjects considered lost to follow-up were counted as treatment failures. This provides for a "worst-case" scenario of treatment efficacy, and the resulting ITT data here is likely an underestimate of actual microwave efficacy for plantar warts, had the patients pursued full courses of treatment and follow-up. Despite the challenging clinic period, the overall number of patients completing full courses of treatment remained suitably high (>50 patients) to allow for reliable statistical analysis; hence efficacy analysis per treatment completion were conducted as well.

Data were tabulated using the Excel® software package and analyzed using one-way ANOVA, paired t-test, and the chi-square test; $p < 0.05$ is considered statistically significant.

3 | RESULTS

3.1 | Patient characteristics

Upon review of the data, a total cohort of 85 patient records (38 females and 47 males), totaling 254 verrucae plantaris were available for inclusion in the review representing a complete, consecutive cohort of cases from March 2019 to December 2020 (Figure 1). The age of the study cohort ranged from 5 to 78 years (mean: 28.6 [SD: 20.7]), with an average of 3.17 verrucae diagnosed per patient (SD: 6.74; range: 1–50), and a mean baseline patient-reported pain score of 3.4 out of 10 (SD: 1.1; range: 2–8). Evidence of treatment recalcitrance, defined as verrucae either persisting for ≥24 months or with inadequate response after ≥2 treatments, was seen in 25.9% (22/85) and 34.1% (29/85) of patients, respectively.[10] Patients, on average, had two or three previous unsuccessful treatments with other modalities prior to receiving microwave. For patients whose treatment response to microwaves was inadequate after the first session, additional sessions were administered with an average intertreatment interval of 1.4 months (SD: 1.1; range 1–7). Post-treatment assessments were conducted at an average of 2.0 months (SD: 2.0; range 1–12) after the last session.

Baseline and disease severity parameters stratified by the number of completed treatment sessions are summarized in Table 1. We observed a higher proportion of recalcitrant verrucae plantaris associated with patients requiring ≥3 treatment sessions; specifically, the proportion of patients with persisting verrucae for ≥24 months increased from 16.7% (5/30) and 11.8% (2/17) to 32.3% (10/31) and

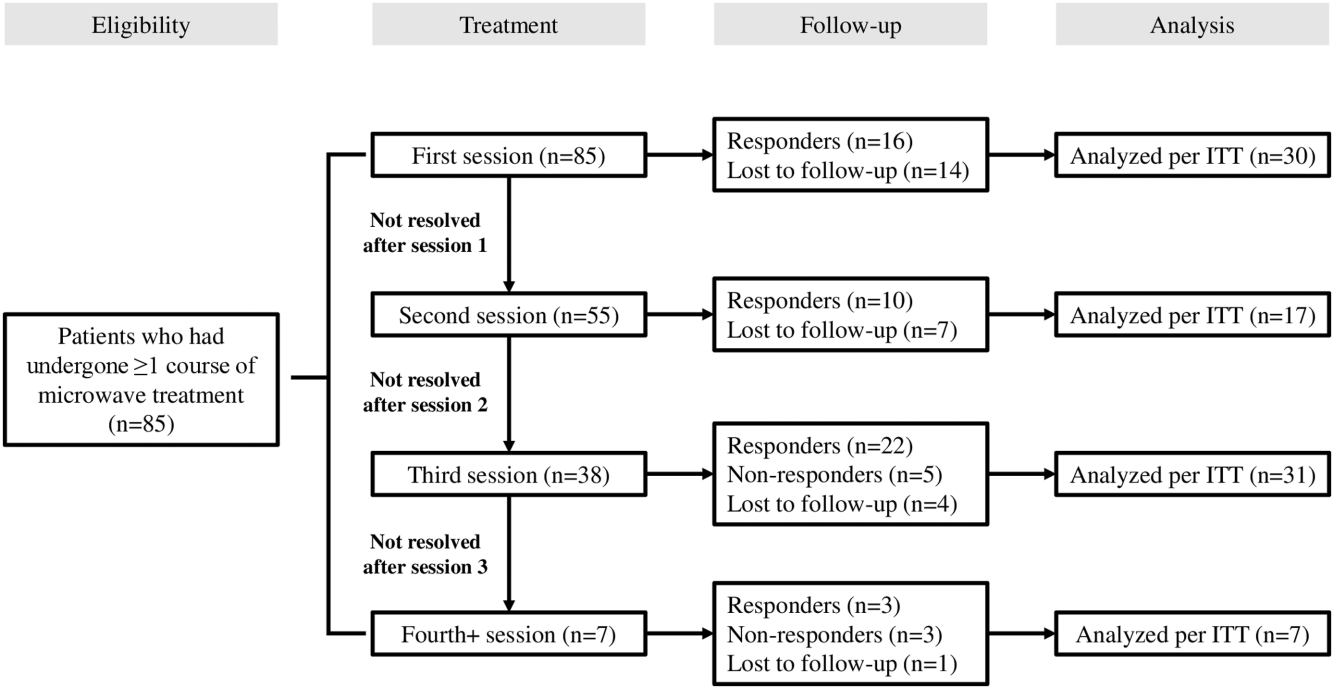

FIGURE 1 Study design. From March 2019 to December 2020, medical records of 85 patients with verrucae plantaris who had undergone one or more sessions of microwave treatment were reviewed. Clinical data were assessed based on the number of microwave therapy sessions administered. Missing data due to lost to follow-up were imputed as nonresponders. ITT, intention-to-treat.

TABLE 1 Baseline characteristics of 85 patients with verrucae plantaris who had undergone one course of microwave treatment ranging from completing one session to four or more sessions.

Parameters	Microwave Treatment			
	1 Session (*n* = 30)	2 Sessions (*n* = 17)	3 Sessions (*n* = 31)	4+ Sessions (*n* = 7)
Female % (*n*)	43.3 (13/30)	41.2 (7/17)	48.4 (15/31)	42.9 (3/7)
Male % (*n*)	56.7 (17/30)	58.8 (10/17)	51.6 (16/31)	57.1 (4/7)
Age, year				
Mean (SD)	23.4 (17.2)	19.5 (16.9)	34.2 (23.4)	47.7 (11.1)
Median (range)	16.5 (6–63)	13 (7–67)	36 (5–78)	53 (24–56)
No. of warts				
Mean (SD)	3.7 (5.6)	2.1 (3.4)	3.4 (8.8)	1.9 (1.1)
Median (range)	2 (1–30)	1 (1–15)	1 (1–50)	2 (1–4)
Duration of lesions % (*n*)				
< 24 months	83.3 (25/30)	88.2 (15/17)	67.7 (21/31)	28.6 (2/7)
≥ 24 months	16.7 (5/30)	11.8 (2/17)	32.3 (10/31)	71.4 (5/7)
No. of prior failed treatments % (*n*)				
< 2 treatments	80 (24/30)	64.7 (11/17)	61.3 (19/31)	28.6 (2/7)
≥ 2 treatments	20 (6/30)	35.3 (6/17)	38.7 (12/31)	71.4 (5/7)
Baseline pain score				
Mean (SD)	3.3 (1.2)	3.8 (1.5)	3.3 (0.9)	3.3 (0.8)
Median (range)	3 (2–7)	3 (2–8)	3 (2–5)	3 (3–5)

71.4% (5/7) for patients requiring one session, two sessions, three sessions or ≥4 sessions, respectively (Table 1). Similar increases were also observed in proportion of patients with histories of two or more prior failed treatments.

3.2 | Efficacy of microwave treatment

Of the 85 patients who received ≥1 microwave session, a total of 59 patients completed their treatment and 26 were lost to follow-up

(30.6% [26/85]). There were 51 patients who achieved the treatment endpoint during follow-up (60.0% [51/85] clearance rate as per intention-to-treat; clearance rate as per treatment completion was 86.4% [51/59]; Table 2). Representative cases of complete and partial resolution are shown in Figures 2 and 3, respectively. Patients required an average of 2.3 sessions (SD: 1.1; range: 1–6) to achieve the treatment end point. Most patients included in this study required up to three treatment sessions over approximately 12 weeks (with the intention to treat clearance rate of 61.5% (48/78); clearance rate per treatment completion was 90.6% [48/53]; Table 2), similar to existing microwave treatment data and other standard wart treatment patterns and time frames reported in the literature (Figure 4).[9–14] Clearance rates (per ITT) were numerically higher in patients who received one or two additional treatment sessions after the primary session (χ^2 [2, $N=78$] = 2.07, $p=0.36$), with up to 71.0% (22/31) achieving the treatment end point after completing the third session, compared with 55.3% (16/30) and 58.9 (10/17) for the first and second session, respectively (Table 3).

In the overall study population, there were no statistically significant differences in the clearance rate of patients with nonrecalcitrant warts versus recalcitrant warts, where recalcitrance was defined by warts lasting for ≥24 months (63.5% nonrecalcitrant [40/63] vs. 50% recalcitrant [11/22]) (χ^2 [1, $N=85$] = 1.24, $p=0.27$), or where recalcitrance was defined by prior inadequate response to ≥2 treatments (58.9% nonrecalcitrant [33/56] vs. 62.1% recalcitrant [18/29]) (χ^2 [1, $N=85$] = 0.079, $p=0.78$). Compared with the overall population, patients requiring between four and seven sessions exhibited a much higher proportion of recalcitrance based on warts lasting for ≥24 months, and prior inadequate response to ≥2 treatments, compared with patients with three or fewer treatments (Table 1); the relatively low response rate of 42.9% (3/7) for 4–7 treatments versus three or fewer treatments could be attributed to continuing or developing recalcitrance in this sample set (Table 3). Most notably, one patient with two verrucae persisting for 84 months, and 11 prior failed treatments, did not achieve the treatment end point after the fourth microwave session. However, another patient with 31 prior failed treatments, and verruca persisting for 24 months, achieved clearance of verrucae plantaris after the fifth treatment session.

Efficacy analysis by age groups found clearance rates for children (≤18 years) and adults (>18 years) at 61.0% (25/41) and 59.1% (26/44), respectively (Table 4). No statistically significant differences were observed (χ^2 [1, $N=85$] = 0.03, $p=0.86$).

3.3 | Pain assessment

Patient-reported wart pain scores at matching pretreatment, during the treatment visit period, and post-treatment time points were available for 53 patients who completed between one and three sessions. The mean wart pain scores were 3.3 (SD: 1.0; range: 2–7), 3.1 (SD: 0.9; range: 0–5), and 0.08 (SD: 0.4; range: 0–3) for pretreatment, during treatment and post-treatment periods,

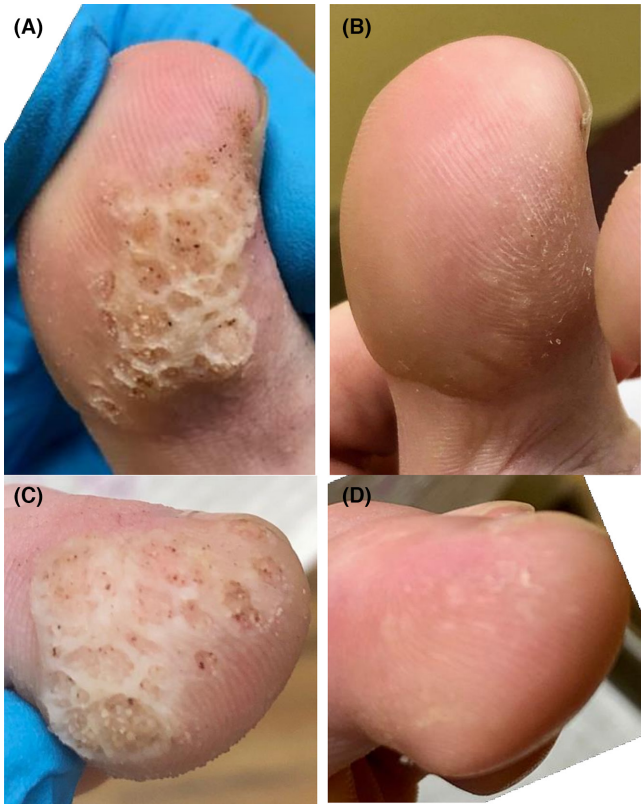

FIGURE 2 Patient presenting with recalcitrant plantar warts achieving complete resolution after microwave treatment. A 14-year-old male, with no known history of diabetes, presented with mosaic lesions on the hallux and second toe of the right foot. Lesions have persisted for 3 years, prior treatments were unsuccessful including salicylic acid, cryotherapy, and imiquimod. The patient subsequently received two sessions of microwave treatment at 4 weeks apart. Photographic assessment of the hallux was conducted at (A, C) baseline and (B, D) 12-week review.

TABLE 2 Efficacy of microwave treatment in patients with verrucae plantaris shown as per standard treatment pattern of up to three sessions, and in all patients who received one or more sessions.

Microwave sessions	Completed/lost to FU (*n*/*n*)	Clearance rate per ITT[a] % (*n*)	Clearance rate per treatment completion[b] % (*n*)
Up to 3 sessions ($n=78$)	53/25	61.5 (48/78)	90.6 (48/53)
≥1 session ($n=85$)	59/26	60.0 (51/85)	86.4 (51/59)

Abbreviations: FU, follow-up; ITT, intention-to-treat.

[a]Missing data were analyzed as "nonresponders" (i.e., treatment failure).

[b]Missing data were excluded from the analysis.

respectively. Compared with pretreatment, no statistically significant changes in pain levels were observed during treatment, while pain levels significantly decreased post-treatment regardless of the number of sessions ($p < 0.0001$; Figure 5). All patients with total clearance of their warts, and all patients who completed one or two sessions, reported pain scores of 0 at follow-up. Two patients who did not achieve clearance after three sessions reported pain scores of 1 and 3.

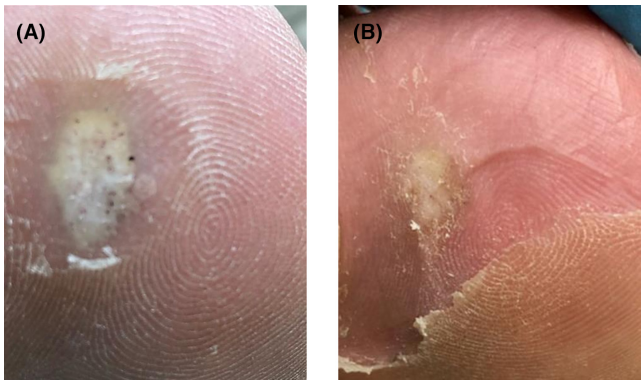

FIGURE 3 Patient presenting with a large recalcitrant plantar wart achieving partial resolution after microwave treatment. A 44-year-old healthy, athletic male, with no know history of diabetes, presented with a large (2.5 × 1 cm) lesion on the right foot. The lesion has persisted for 2 years, prior treatments consisting of salicylic acid, cryotherapy, imiquimod, debridement, and needling were unsuccessful. The patient subsequently received four sessions of microwave treatment at 4 weeks apart, and two additional sessions at 8 weeks apart. Photographic assessment was conducted at (A) baseline and (B) 36-week review.

3.4 | Safety

One adverse event of ulceration, following the administration of local anesthetic prior to microwave treatment, was reported. A previous survey study of patients who had received Nd:YAG laser for wart treatment reported an ulceration rate of 40% (4/10) in patients who underwent local infiltration of the lesion prior to laser application.[15] It is possible that injections of anesthetic in the tissue increases local fluid content, which creates a greater target for microwave with subsequently increased heating of the area undergoing treatment. Additionally, patient feedback on pain would be absent, leading to an increased risk of overtreatment, and hence ulceration. In the current study, only one patient received pretreatment local anesthesia. This patient subsequently developed ulceration at the treatment site, which led to the cessation of any local anesthesia administration at subsequent visits and no further ulceration was observed. There were no other adverse reactions.

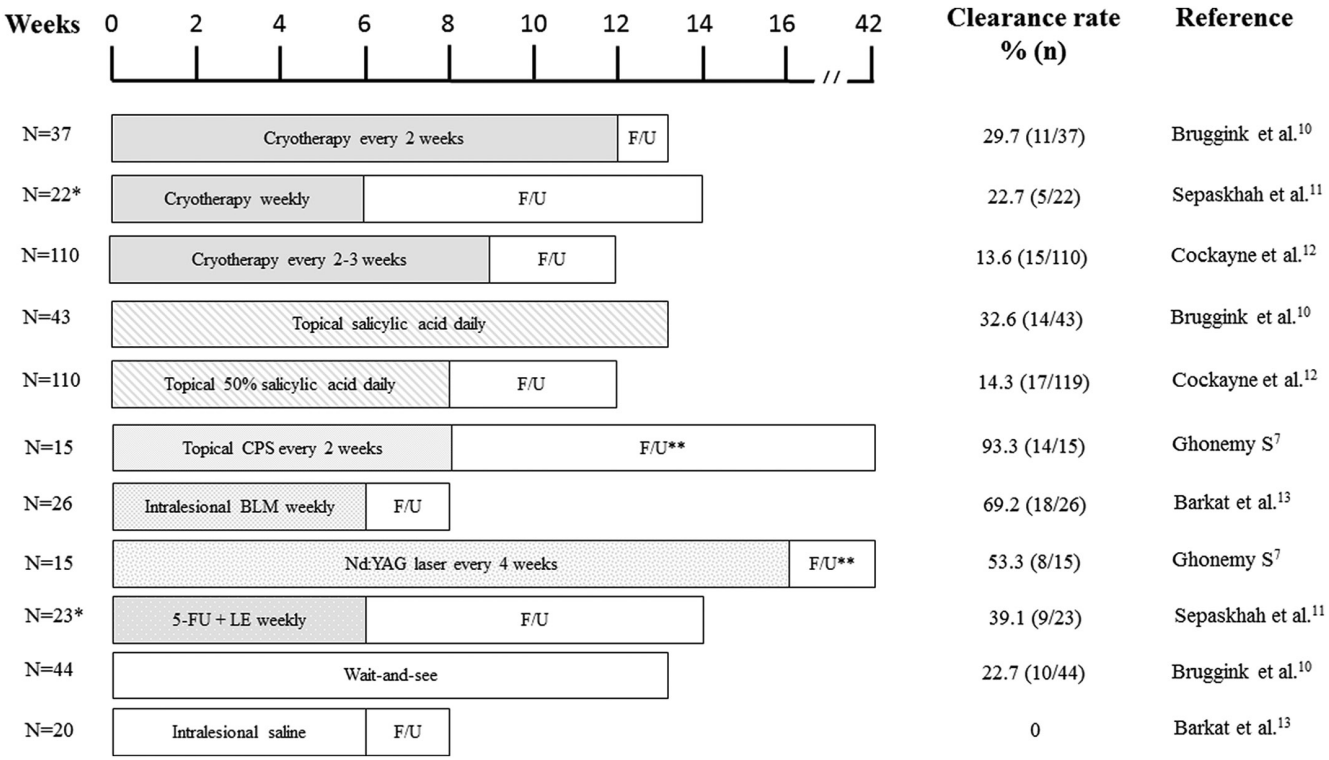

FIGURE 4 Previously reported clearance rates of other treatment modalities for verrucae plantaris. Clearance rates, defined as the complete visible clearance of warts with or without dermoscopic evaluation, were extracted from five studies treating patients with cryotherapy, salicylic acid, 5-fluorouracil, and/or bleomycin.[10–14] Data from matching control or placebo groups were also extracted if available. *Mixed patient cohort of common and plantar warts. **Follow-up assessment was conducted 6 months after the end of study. BLM, bleomycin; CPS, 1% cantharidin, 20% podophyllin resin, and 30% salicylic acid; F/U, follow-up; 5-FU + LE, intralesional 5-fluorouracil injection admixed with lidocaine and epinephrine.

Microwave sessions	Completed/lost to FU (*n*/*n*)	Clearance rate per ITT[a] % (*n*)	Clearance rate per treatment completion[b] % (*n*)
1 Session (*n* = 30)	16/14	55.3 (16/30)	100.0 (16/16)
2 Sessions (*n* = 17)	10/7	58.9 (10/17)	100.0 (10/10)
3 Sessions (*n* = 31)	27/4	71.0 (22/31)	81.5 (22/27)
4+ Sessions (*n* = 7)	6/1	42.9 (3/7)	50.0 (3/6)

Abbreviations: FU, follow-up; ITT, intention-to-treat.

[a]Missing data were analyzed as "nonresponders" (i.e., treatment failure).

[b]Missing data were excluded from the analysis.

TABLE 3 Efficacy of microwave treatment in patients with verrucae plantaris stratified by the number of treatment sessions administered.

TABLE 4 Efficacy of microwave treatment in patients with verrucae plantaris stratified by age groups.

Age group	Completed/lost to FU (*n*/*n*)	Clearance rate per ITT[a] % (*n*)	Clearance rate per treatment completion[b] % (*n*)
≤18 years (*n* = 41)	26/15	61.0 (25/41)	96.2 (25/26)
>18 years (*n* = 44)	33/11	59.1 (26/44)	78.8 (26/33)

Abbreviations: FU, follow-up; ITT, intention-to-treat.

[a]Missing data were analyzed as "nonresponders" (i.e., treatment failure).

[b]Missing data were excluded from the analysis.

4 | DISCUSSION

The results from this work demonstrate a significant clearance rate of verrucae plantaris in a group of patients of varying age, disease severity, and treatment histories, using microwave therapy. We found that 60% (51/85) of patients achieved clearance after an average of 2.3 sessions, and the rate was higher in patients who completed three sessions (71.0% [22/31]). However, these ITT results are a "worst-case" estimate which likely underestimates the true microwave response potential, based on comparison with the clearance rates per treatment completion. Nonetheless, the ITT results compare favorably to other treatment modalities (Figure 4), and corroborated findings from a pilot UK study treating plantar warts with up to four microwave sessions.[10–14,16] In contrast to patients treated with cryotherapy or salicylic acid, we found no significant differences in clearance rates between children and adults (61.0% [25/41] vs. 59.1% [26/44]).[13] Other factors potentially affecting treatment outcomes may include the number and size of lesions, inclusion of patients with recalcitrant warts, hands warts or history of self-treatment, as well as energy settings of the microwave device.[9,13,16]

Analysis of the patient's wart pain scores revealed that all patients, regardless of final outcome, experienced significant pain reduction post-treatment whereby average pain scores reduced from 3.3 to 0.08 (Figure 5). The effect of microwave reducing verrucae pain was demonstrated in a similar manner in the UK study with a reduction in the mean pain scores noted prior to and at the conclusion of treatment (2.85 to 0).[16] A similar reduction in pain has also been observed in patients undergoing microwave treatment for their painful corns.[17] The reasons for this effect are unclear although it has been suggested that heating of the nerve endings in the skin may lead to nerve desensitization with an increase in the pain threshold, leading to a subsequent drop in pain experienced by the patient.[18] Similarly, microwave-induced hyperthermia has been reported to give pain relief to patients with tendinopathy, osteoarthritis, or carpal tunnel syndrome, further supporting the notion that microwave treatments may exert an effect on local pain mechanisms.[19–21]

A previous survey found more than half of physicians (66.2% [47/71]) apply pain mitigation measures during microwave treatment sessions, including anesthesia, gating or distraction techniques, as well as nitrous oxide.[9] In this study, we observed no significant increases in pain levels at the lesion site during treatment (Figure 5). This difference may be attributed to the energy setting of the microwave device (8 W for 2 s corresponding to 16 Joules in this study compared with 16–20 Joules as previously reported).[9] One case of ulceration at the treatment site was reported in a patient who received a local anesthetic, and this observation led to the cessation of local anesthesia at the physician's discretion. No other patient received local anesthesia for the duration of this study, and no additional cases of ulceration were reported. Other previously reported adverse reactions in response to microwave treatment not observed in this study included poor healing and blistering.[9]

Comparison with previous reports on traditional treatment modalities, such as cryotherapy and salicylic acid, as well as investigational agents including bleomycin and 5-fluorouracil, showed varying degrees of efficacy in inducing the complete clearance of plantar warts (Figure 4). The clearance rate of plantar warts in our study was 60.0% (51/85), which is notably higher compared to patients treated with cryotherapy (13.6% [15/110] to 29.7% [11/37]) or topical salicylic acid (14.3% [17/119] to 32.6% [14/43]).[11–13] Intralesional bleomycin injection, although effective with 69.2% (18/26) of patients achieving clearance at week 8, may have limited utility due to pain at the injection site; especially for patients with multiple warts.[12,22] With intralesional bleomycin injection one case of hemorrhagic eschar was also reported.[22] Similarly, another study reported a 93.3% (14/15) clearance rate in patients treated using a solution of 1% cantharidin, 20% podophyllin resin, and 30% salicylic acid following surgical delamination of plantar warts.[10] Its side

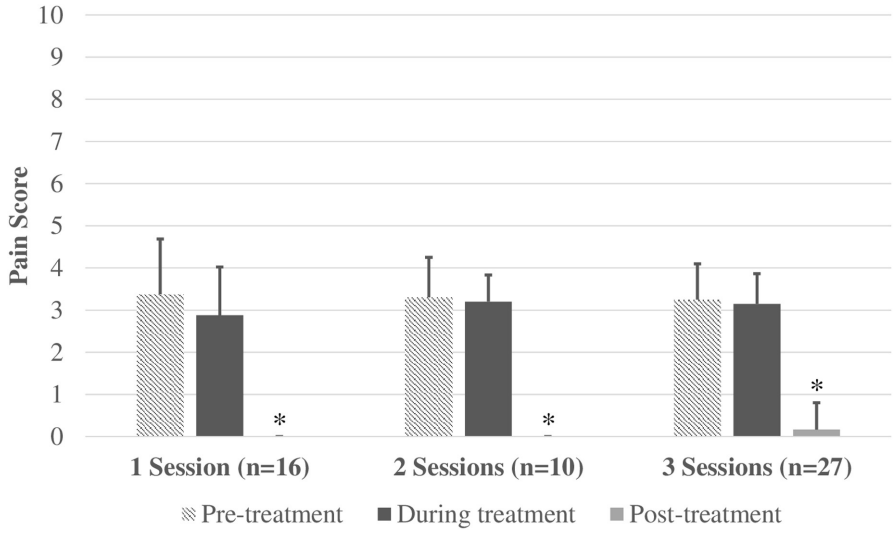

FIGURE 5 Pain scores reported in patients completing up to three microwave treatment sessions at pretreatment, during treatment, and post-treatment time points. * $p < 0.0001$ compared with pretreatment.

effects profile, including pain, bulla, and hemorrhagic bulla, may also limit its utility.[10]

The high clearance rate demonstrated by the microwave application may arise as an effect of heating HPV infected cells. The unit delivers microwave radiation into the skin which leads to the rapid elevation of the treated area into the hyperthermic or heat shock range of 41–45°C, a few degrees above body temperature (37°C). Previous research has shown that elevating body tissues to within this range can modulate a range immune processes without causing significant tissue damage.[23] Hyperthermic temperatures applied to HPV infected skin can amplify wart resolution by positively affecting many facets of the immune response.[24] If tissue is exposed to temperatures above 41°C for prolonged periods, cell damage and death is likely. However, cells placed under stress from microwave heating of a few seconds produce molecules known as heat shock proteins (HSPs).[25] These have evolved to protect cells in extreme stress conditions from cell death. HSPs have a number of functions—first and foremost as protein chaperones that are involved with the folding, shape regulation, and degradation of intracellular proteins.[6] Moreover, HSP-70 has been shown to induce the maturation of Langerhans cells and enhance their migration to the lymph nodes. When comparing normal skin to HPV infected skin, it was discovered that the migratory response was more marked in the HPV infected skin.[26] HSP release also has been shown to stimulate cytokine release from antigen presenting cells, as well as nitric oxide, chemotactic factors from macrophages and to stimulate antitumor activity by the immune system.[23] Additionally, microwave treatment appears to have a more direct effect on HPV infected keratinocytes by disrupting viral protein expression and cell apoptosis.[25,27]

Some limitations of our study include the retrospective study design and lack of head-to-head comparisons with other treatment modalities. This study is among the first to assess the utility of microwave devices in patients with verrucae plantaris, including those with prior failed treatments and lesions persisting for ≥2 years characteristic of recalcitrant warts. Further studies are warranted to confirm our findings, and to further elucidate the mechanism of action of microwave treatment.

5 | CONCLUSIONS

The application of microwave energy in the treatment of verrucae plantaris has demonstrated a high clearance rate of 60% (51/85) in a cohort of 85 patients following one course of treatment. The clearance rate was 86.4% (51/59) in patients who completed treatment. Patients who received three sessions achieved a higher clearance rate (71.0% [22/31]). Clearance rates were similar between children (61.0% [25/41]) and adults (59.1% [26/44]). A notable number of patients with recalcitrant warts also achieved clearance after four or more sessions (42.9% [3/7]). Significant improvements in pain associated with lesions were seen in patients who completed treatment (post-treatment pain score of 0.08 [0.4], $p < 0.0001$ relative to pretreatment); no other adverse events were observed. Microwave treatment, in this series of patients, has demonstrated to be a safe and effective means of treating verrucae plantaris, where other treatment modalities may have failed or demonstrated poor efficacy.

AUTHOR CONTRIBUTIONS

IRB, RMC, and AKG were involved in conceptualization; TW, EAC, IRB, and RMC were involved in data curation; TW, EAC, IRB, and RMC were involved in formal analysis; RMC was involved in investigation; RMC, AKG, IRB, EAC, and TW were involved in methodology; AKG and EAC were involved in project administration; AKG and RMC were involved in resources; AKG, EAC, and IRB were involved in supervisions; AKG and RMC were involved in validation; TW was involved in visualization; TW, RMC and IRB were involved in writing—original draft preparation; AKG and EAC were involved in writing—review and editing.

CONFLICT OF INTEREST STATEMENT

AKG, TW, and EAC report no competing interests to declare. IRB is a consultant for Emblation Limited, UK. RMC is a consultant for Emblation Inc, USA.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL STATEMENT

Not applicable. The authors declare that human ethics approval was not required for this article.

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