Project: **SEAWave**

SCC incidence and multiplicity

Work Package:	WP6
Deliverable:	D6.3
Deliverable No.:	D20

Abstract

Deliverable 6.3 is related to the evaluation of the impact of 5G exposure on Squamous Cell Carcinoma (SCC) development in adult DMBA-initiated CarS mice exposed to FR2 for 100 days.

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Contents

1 Introduction

The carcinogenesis-susceptible (Car-S) mouse line is a well-established animal model used to study squamous cell carcinoma (SCC), the second most prevalent form of skin cancer. This model was developed at ENEA through 19 generations of bidirectional selective breeding focused on susceptibility to two-stage skin carcinogenesis.

In this model, the mice are subjected to initiation with 9,10-dimethyl-1,2-benzanthracene (DMBA), a potent carcinogen. This step introduces irreversible genetic changes in the multipotent progenitor cells or stem cells of the skin, initiating the carcinogenesis process. Following this, the mice are treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a potent inflammatory chemical, administered twice weekly for 100 days. The result is 100% papilloma incidence, with a high average tumor multiplicity of papillomas per mouse. This two-step process of initiation and promotion mirrors how human skin cancers, particularly SCC, often develop—starting with genetic mutations in stem cells, followed by clonal expansion under the influence of tumor promoters. The promotion step of the carcinogenesis model is not only to foster tumor growth but also to establish a pro-inflammatory microenvironment that supports the clonal expansion of genetically altered skin cells.

Some studies on lower radiofrequency exposure (such as 2G, 3G, 4G, and early 5G frequencies) than the frequency studied in this experiment, have suggested that exposure to electromagnetic fields (EMFs) could trigger inflammatory responses. The mechanisms proposed include the generation of reactive oxygen species (ROS), which can induce oxidative stress, and changes in cell signaling pathways, including those that regulate inflammation. For 5G FR2, which operates at higher frequencies (millimeter waves, around 24 GHz to 100 GHz), there is still a lack of evidence.

Given that the skin plays a crucial role in immune response and environmental adaptation and is a direct target of 5G radiation at high frequencies, Car-S mice provide a reliable model to explore whether prolonged exposure to 5G, at a frequency of 27.5 GHz, may contribute to the development of skin cancer. If 5G radiation were to act as a promoter in the two-stage carcinogenesis model, it could lead to enhanced clonal expansion of genetically altered skin progenitor cells, contributing to tumor growth.

2 Material and Methods

We conducted a controlled study using 8-week-old Car-S mice (n=140, 70 males and 70 females). Mice received a single dose of DMBA (5 µg in 100 µl acetone) three days before the start of the exposure.

Whole-body exposure to 27.5 GHz radiation was administered at two distinct power densities: 6.67 W/m^2 (Low-Power Density, LPD) and 20 W/m^2 (High-Power Density, HPD). Mice were randomly assigned to one of three experimental groups: HPD (n=36), LPD (n=36), or SHAM (n=36), thus exposed for 100 days in three different, specifically designed, reverberation chambers under blind conditions, which was revealed only at the end of all the procedures of data analysis for the different endpoints.

As a positive control for tumor initiation, the remaining DMBA-initiated Car-S mice underwent a classical two-stage carcinogenesis protocol. In this group, TPA (1 µg in 100 µl acetone) was applied topically twice a week for 100 days, starting seven days after DMBA treatment. Tumor incidence (percentage of mice with at least one papilloma) and tumor multiplicity (average number of papillomas per mouse) were recorded twice weekly.

After 100-day exposure, dorsal skin samples were collected and snap-frozen for molecular analysis or fixed in 4% formalin.

Dorsal skin samples were processed using standard histological techniques. Four-micrometer-thick sections were dewaxed and stained with hematoxylin and eosin (H&E) for routine examination. For immunostaining, skin sections were incubated with a primary antibody against Ki67, following the manufacturer's guidelines, then treated with a secondary anti-rabbit antibody. The antigen-antibody complex was visualized using DAB, and images were analyzed with HistoQuest software.

To assess skin inflammation, total RNA was extracted from snap-frozen dorsal skin samples using the RNeasy Microarray Tissue Kit. qPCR was performed to analyze the expression of genes previously identified as deregulated in neonatal mice after short-term exposure (21 days; see for details Deliverable No. D19).

3 Results

3.1 DMBA initiation/TPA promotion

The efficacy of DMBA initiation was thoroughly validated through two key observations. First, a 100% incidence of papillomas was observed within 50 days in the DMBA/TPA positive control group, confirming the expected outcome of the treatment. Second, the papilloma multiplicity curve followed the anticipated pattern, further reinforcing the reliability of the model in inducing papilloma formation. These results collectively support the effectiveness of DMBA as an initiator in the experimental setup. (**Figure 1**).

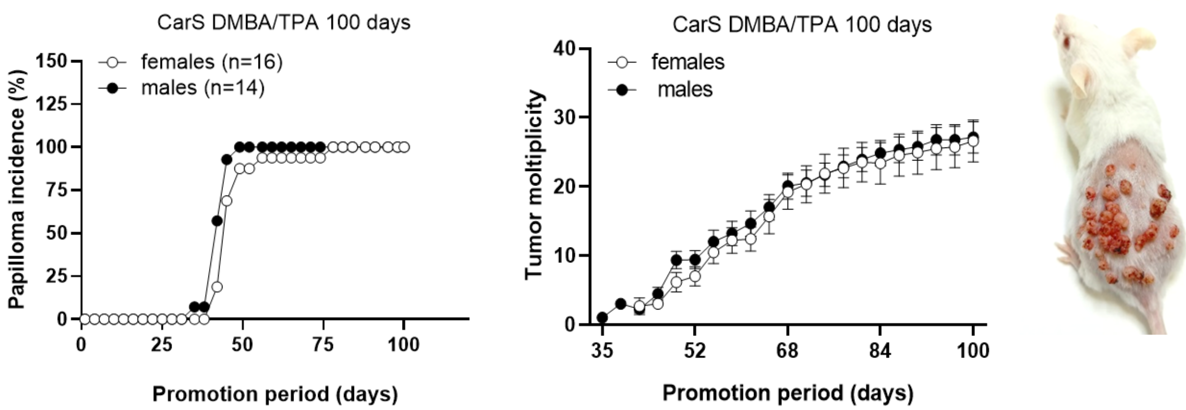

Figure 1. Two-stage chemical carcinogenesis. Tumor incidence in DMBA/TPA positive control group (Ctr+) was expressed as a percentage of mice developing at least one papilloma. Tumor multiplicity was expressed as an average number of tumors per mouse. Representative image of papilloma appearance in the positive control group (Ctr+).

3.2 DMBA initiation/5G FR2 promotion

Throughout the 100-day exposure period with 5G, no significant signs of distress or illness were observed in any of the exposed mice. However, two males from the LPD group and two from the HPD group were excluded due to aggressive behavior among cage mates.

A significant reduction in body weight was observed in male mice in the HPD group compared to the LPD and SHAM groups (**Figure 2**), but no such effect was seen in female mice. The underlying cause of this result remains unclear and may be attributed to several factors, including increased energy expenditure, metabolic changes, or stress responses induced by high-power 5G exposure. Additionally, hormonal or physiological differences could offer a protective effect against weight changes in female mice compared to males. Further dedicated experiments are needed to address these questions and validate the findings.

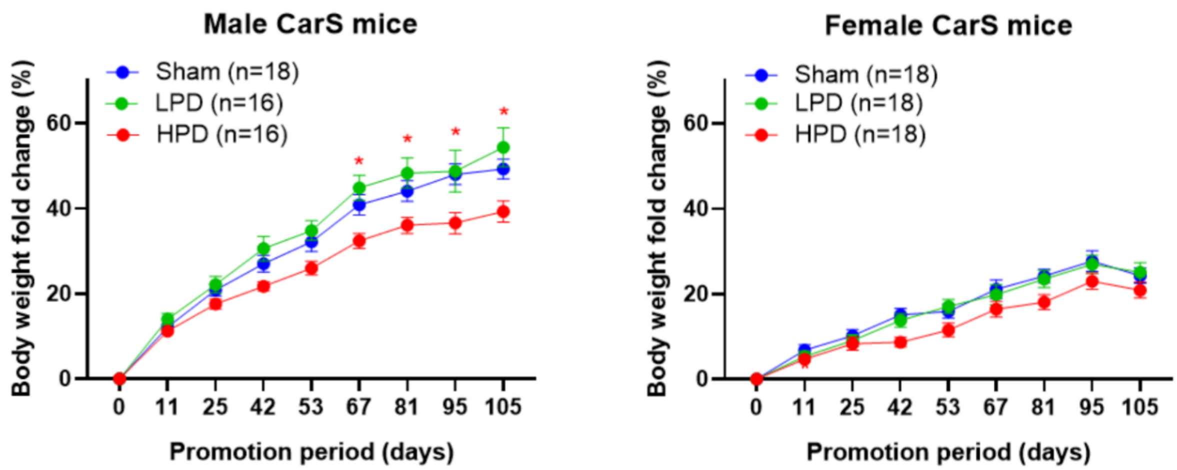

Figure 2. Body weight expressed as fold change in the comparison between LPD (green) and HPD (red) versus SHAM (blue) in Car-S male and female mice.

Notably, after 100 days of 5G exposure, no visible papillomas were detected in either the HPD or LPD exposed groups. The skin of all the animals in the experiment, regardless of sex, appeared normal, and no abnormalities such as dryness, redness, alopecia, etc., were observed (**Figure 3**).

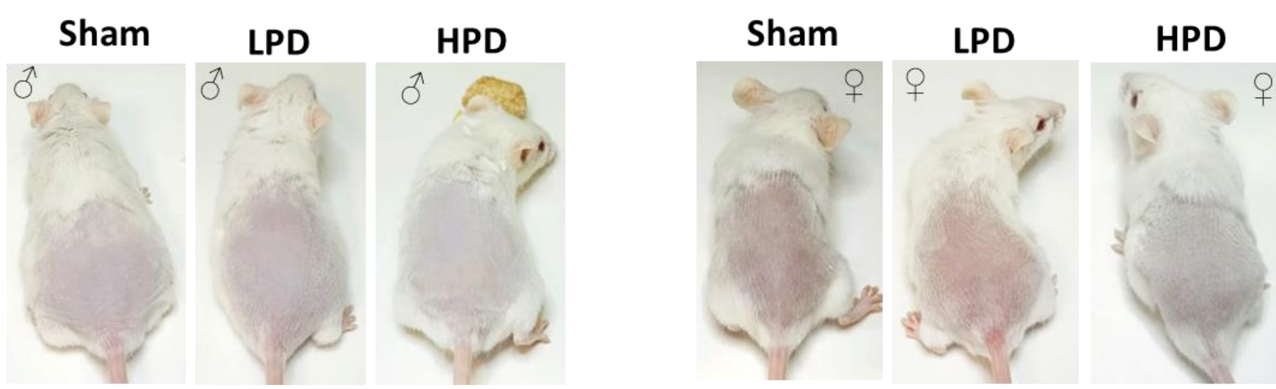

Figure 3. Representative images of the skin after 100 days of 5G exposure.

We also investigated the proliferation index of the epidermis in the mouse cohorts (LPD, HPD, and SHAM) to exclude the presence of early phases of papilloma development, which are not evaluable macroscopically. To this end, 75 skin samples (~25 per experimental group) were analyzed for Ki67 immunostaining (**Figure 4A**). Between 5 and 8 frames per sample were acquired at 20× magnification, and only basal layer cells in the interfollicular epidermis were counted. The results showed no significant changes in basal layer cell proliferation (**Figure 4B**), and no proliferative lesions were detected.

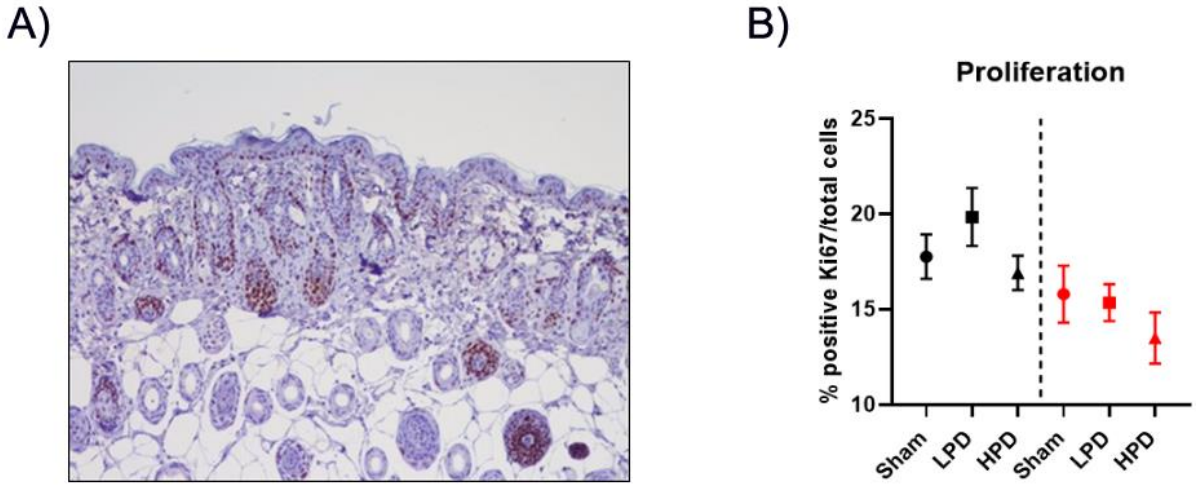

Figure 4. Proliferation index of the epidermis. A) Representative image illustrates Ki67 immunostaining. **B)** Proliferation index is expressed as the percentage of Ki67-positive cells in the interfollicular epidermis relative to the total number of counted cells. Male mice are represented in black, while female mice are represented in red.

Task 6.5 results, as detailed in Deliverable No. D19, showed that 5G exposure during neonatal mouse development caused significant dysregulation in inflammatory gene expression and nociceptor activation. Specifically, *Ccl4*, *Tnfsf11*, and *Csf2* were upregulated, while *MrgprD* and *Cgrpα* genes in cutaneous nociceptors were downregulated. *MrgprD* is involved in itch, pain, and stress, while *Cgrpα* plays a role in nociception and neurogenic inflammation. This downregulation was linked to increased mast cell degranulation and activation of inflammatory mediators (*Il4, Il13, Csf2*), indicating a strong inflammatory response triggered by 5G exposure in newborn mice.

To assess whether similar effects occur in adulthood, we examined the same gene set in the skin of Car-S adult mice exposed to 5G for 100 days. Unlike in young mice, a clearly different pattern of dysregulation in inflammatory genes characterizes the adult skin at the end of the exposure. As shown in **Figure 5A**, *Ccl4* was significantly downregulated under LPD exposure in both males and females, as well as in HPD-exposed females; similarly, *Tnfsf11* and *Csf2* were downregulated under LPD conditions in both sexes. Analysis of cytokines (**Figure 5B**) released by mast cell degranulation revealed that *Il4* was downregulated in both males and females under LPD conditions, while *Il13* showed a similar downregulation in LPD exposure but was induced under HPD exposure. Unlike in young mice, skin nociceptor markers were not consistently downregulated in adults. As shown in **Figure 5C**, *MrgprD* expression significantly increased in LPD-exposed males and *Cgrpα*, which was downregulated in young mice after 21 days of exposure, showed a similar trend after 100 days in adults—except in HPD-exposed females, where its expression did not follow the same pattern.

These findings suggest that prolonged exposure to 5G radiation or the skin's different response due to age may differentially influence the inflammatory responses and nociceptors gene expression in adult Car-S mice compared to newborn *Ptch1*$^{+/-}$ mice.

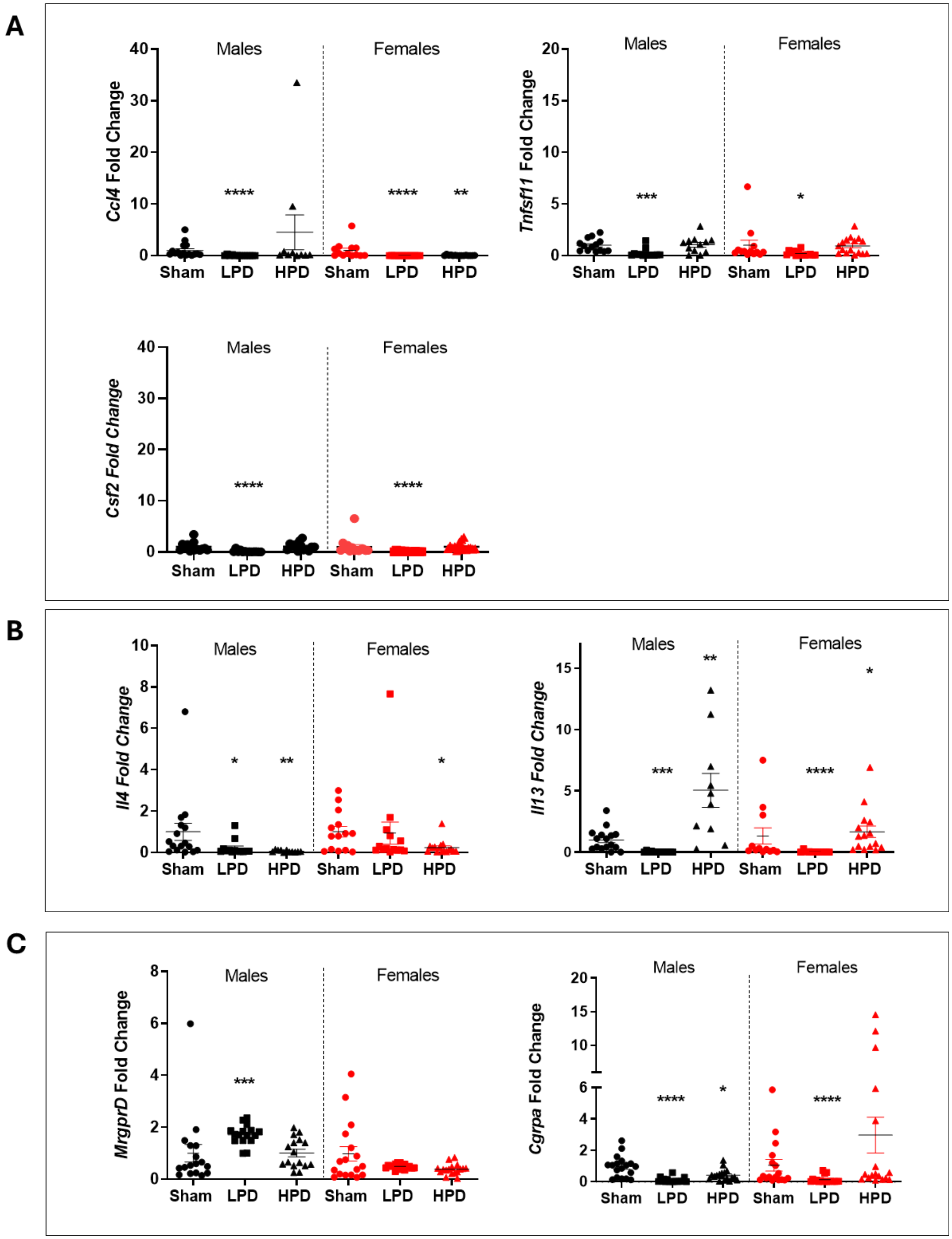

Figure 5. Evaluation of gene expression. **A)** Inflammatory genes: *Ccl4*, *Tnfsf11*, *Csf2*. **B)** Cytokines released by mast cell degranulation: *Il4*, *Il13*. **C)** Gene involved in nociception: *MrgprD*, *Cgrpα*. Mann-Whitney U test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

4 Conclusions

Our findings suggest that prolonged exposure to 5G does not induce any significant evidence of SCC development in the skin, abnormal cell proliferation, or the activation of nociceptors and inflammatory responses in the skin of Car-S mice. This indicates that, under the conditions tested, 5G exposure does not seem to result in overt pathological changes at the cellular or tissue level. However, notable variations between LPD and HPD exposure conditions in several key endpoints - such as body weight changes and markers of inflammation - underscore the possibility of dose-dependent effects, where higher levels of exposure might lead to subtle but significant biological alterations. The observation that HPD impairs body weight gain in male mice underscores the importance of further research to uncover the biological mechanisms driving these effects.

Moreover, differences in the gene expression profiles of inflammatory-related genes following 5G exposure in young *Ptch1*$^{+/-}$ and adult Car-S mice provide valuable insights into the complexity of the immune response to 5G radiation. These findings suggest that susceptibility to inflammatory responses and nociceptive alterations may be shaped by several factors, including genetic background, age at the time of exposure, and the duration of the exposure itself. Such variations emphasize the need for a deeper understanding of how these factors interact and contribute to the overall biological impact of 5G exposure.

Looking ahead, on-going experiments focusing on the long-term (6-month) biological effects of 5G exposure (Task 6.4: Deliverable No. D21 “BCC incidence and multiplicity”), particularly across different developmental stages and varying exposure intensities, will be crucial in providing further clarity on the potential risks associated with prolonged 5G radiation. In particular, the use of the *Ptch1*$^{+/-}$ mouse model could offer valuable insights into how prolonged exposure might influence cancer-related pathways, inflammation, and sensory changes across the lifespan.

The results of these experiments will be presented at the next annual meeting of the Bioelectromagnetic Society (BioEM 2025), in Rennes, France, from 22 to 27 June.