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NADPH OXIDASE AND UNCOUPLED NITRIC OXIDE SYNTHASE ARE MAJOR SOURCES OF REACTIVE OXYGEN SPECIES IN ORAL SQUAMOUS CELL CARCINOMA. POTENTIAL IMPLICATIONS FOR IMMUNE REGULATION UNDER HIGH OXIDATIVE STRESS CONDITIONS.

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The development of cancer is associated with high oxidative stress and at the same time with immune system activation. Tumors develop efficient mechanisms of protection against the immune response, which allow them to escape the immune surveillance. Simultaneously, key events in the process of carcinogenesis are related to oxidative stress. The relationship between the two remains unknown. Novel understanding of oxidative stress shows that discrete changes of activities of certain enzyme systems such as NADPH oxidases or nitric oxide synthases may be more important than the overall balance of production and removal of reactive oxygen species. Such imbalance of nitric oxide and superoxide production could modify inflammation and immune regulation. We studied superoxide anion production (by lucigenin enhanced chemiluminescence - 5 μM), NADPH oxidase activity and nitric oxide synthase (NOS) dysfunction. In parallel mRNA expression of immunomodulatory markers such as FoxP3 (T regulatory cell marker), CCR6 (mucosal homing effector T cell marker) and CD85j (NK cell/CD8 T cell Ig-like MHC class I inhibitory receptor) was determined. Basal superoxide production and NADPH oxidase activity are increased in oral squamous cell carcinoma. Tumor superoxide production was inhibited by NADPH oxidase inhibitor apocynin and by NOS inhibitor L-NAME. This indicates, for the first time, that oral squamous cell carcinoma is characterized by dysregulated nitric oxide synthase, which apart from increased NADPH oxidase activity contributes to oxidative stress and may be related to the immuno-pathology of these tumors. Studied tumors were infiltrated by CCR6+, but showed lower expression of both CD85j and FoxP3 mRNA. Finally, the CD85j mRNA expression was inversely correlated to oxidative stress parameters.

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These preliminary studies indicate that tumor oxidative stress, related to NADPH oxidase activity and NOS activity could be related to immune responses to cancer, thus therapeutic modification of oxidative stress, which could include the correction of NOS dysfunction, could facilitate immune surveillance.

**Key words:** Immune regulation, superoxide, nitric oxide, inflammation, oxidative stress, NADPH oxidases, eNOS, cancer, epithelium

**INTRODUCTION**

Squamous cell carcinoma (SCC) is the most common form of oral cancer, accounting for the nearly 90% of all cases of oral cancer (1). The pathogenesis of this form of cancer is complex and in spite of large amount of research, the treatments available have relatively poor effects on prognosis, when compared to other forms of cancer (1).

Carcinogenesis is the phenomenon when the host cell after DNA mutations escapes from the organism regulation pathways and becomes uncontrolled dividing cell with the ability of invading other tissues by a local growth or distant cells penetrating by metastasis (2). However, cancers are not only autonomous masses of mutant cells but they are complex and well organized structures consisting of different pathologically changed cells such as fibroblast, epithelial cells, innate and adaptive immune cells and other cells that are unique to each tissue microenvironment (2). The development of cancer is associated with immune system activation, although the response is greatly inefficient which allows the tumor to escape the immune surveillance (3, 4). However in some cases immune responses to pathogens may actually lead to oxidative stress and inflammation leading to the development of cancer. Oral and GI tract infections with *Helicobacter pylori* may be a good example of such conditions (5 - 10).

Key events in carcinogenesis are related to oxidative stress and oxidative modification of protein and DNA (11). It has been recently understood however that in oxidative stress, discrete changes of activities of certain enzyme systems such as NADPH oxidases or nitric oxide synthases may be more important than the overall balance of production and removal of reactive oxygen species (ROS) (12). In such dysfunctional state enzyme system would produce a pathological product instead of the normal product. Nitric oxide synthase is a good example of such pathology. In certain conditions, such as lack of substrate or co-factor (tetrahydrobiopterin) NOS can produce free radicals, mainly superoxide anion (12). Thus such enzyme would produce pathogenic superoxide instead of normally physiologically generated nitric oxide (12). The potential importance of these changes remains undefined in relation to cancer pathogenesis and pathophysiology. Such changes would also be able to modify
not only the function of cancer cells themselves, but also could affect angiogenesis as well as regulate immune responses to cancer (3, 4), as they are greatly regulated by the balance between nitric oxide and superoxide (13). Thus it is important to determine the role of the disturbances within NADPH oxidases as well as nitric oxide synthases for the immune regulation in the conditions of high oxidative stress. In order to approach this problem we used tissues obtained from oral cancers, as these tissues are known to represent a very high oxidative stress environment.

We describe for the first time, that oral cancers show features of dysregulated nitric oxide synthase, apart from increased NADPH oxidase activity and that it may be related to the immunopathology of these tumors. The immune system parameters addressed in this study include the Foxp3 gene as a marker of T regulatory cell infiltration of the cancer tissue, CCR6 expression - a mucus and skin homing receptor allowing to estimate the extent of the immune system reaction to the cancer and finally the CD85j, Ig-like MHC class I inhibitory receptor which participates in self tolerance on NK and CD8 T cells by raising the threshold of their activation. Our pilot studies suggest that oxidative stress parameters are particularly strongly inversely related to CD85j expression. Further studies of larger populations are needed to determine the exact relationship between the immune system activation and the characteristics of oxidative stress in the setting of cancer.

MATERIALS AND METHODS

Samples of oral cancer:

Segments of oral cancers not needed for clinical and diagnostic procedures were collected on ice-cold PBS saline and immediately transported to the laboratory where they were studied or stored in -80°C for further determination of mRNA levels. Samples were obtained from subjects undergoing surgical excision of the disease including cancer of the tongue (30%), retromolar triangle (40%), gingivae (20%), floor of the mouth (oral fundus) (10%) Control samples were otherwise discarded segments of mucosa or muscle were obtained from other surgeries. Study was approved by the Local Ethics Committee of Jagiellonian University and informed consent was obtained.

Determination of superoxide production

Superoxide production was measured in segments of tumor tissue using 5μmol/L lucigenin-enhanced chemiluminescence as previously described (14, 15, 16, 17, 18). Briefly, 50 mg wet weight segments of tissue were placed in Krebs-HEPES buffer (pH 7.4; in mmol/L: NaCl 99.01, KCl 4.69, CaCl₂ 1.87, MgSO₄ 1.20, NaHEPES 20.0, K₂HPO₄ 1.03, NaHCO₃ 25.0, and D(+)-Glucose 11.1) and were dark-adapted and oxygenated. After dark adaptation, sample was transferred to a scintillation vial containing 5μmol/L lucigenin. After dark adaptation, basal chemiluminescence was measured using a Berthold FB12 Lumimeter modified to maintain 37°C, for approximately 10 min until stable level of production was detected.
NADPH oxidase activity was measured following stimulation in the presence of 100 μmol/L NADPH (Sigma Aldrich) until plateau phase of detection (about 20 min). Specificity for superoxide of this assay has been confirmed by pre-incubation of tissues with superoxide dismutase.

Data were expressed as relative light units (RLU) per second per milligram of dry weight. Dry weight was determined by exposing tissues to 56°C for 24 h.

In separate experiments inhibitors of nitric oxide synthase (N-[omega]-nitro-l-arginine methyl ester, L-NAME, 300 μmol/L) and NADPH oxidase (Apocynin; 100 μmol/l) were added to tissue samples for 20 min at 37°C prior to chemiluminescence measurements (19).

**Sample preparation and RNA extraction**

The tissues were stored at -80°C until RNA extraction. 30 mg of tissue was thawed trizol (Tri reagent, MRI, US) and homogenized using mechanical followed by dounce homogenization. RNA was extracted using manufacturers protocol, standard for phenol-chloroform extraction. Following Trizol extraction, RNA was re-purified using a commercially available kit (RNeasy Mini Kit, Qiagen Inc., USA) and quantified spectrophotometrically (absorbance at 260 nm). Total RNA (1 μg) was subsequently used for reverse transcription (RT) in a final reaction volume of 20 μL (as described previously (20)).

**Reverse Transcription**

The template (cDNA) was generated from a reverse transcription reaction and the procedure for cDNA generating was based on High Capacity Reverse Transcription kit (Applied Biosystems, USA). Briefly, total RNA from each sample was reverse-transcribed using random hexamers and MultiScribe Reverse Transcriptase in a final reaction volume 20 μL. The reaction was accomplished in a thermal cycler at 37°C for 120 min and after first-strand cDNA synthesis the reaction was stopped by heat inactivation in 85°C for 5 min.

**Real-Time Polymerase Chain Reaction**

The cDNA was subjected to quantitative PCR using SYBR®Green PCR Quantitee kit (Quiagen) and Roche Lightcycler 3500 fluorescent real-time PCR system (Roche). Mg2+ concentrations were 1.5 mmol/l. Annealing temperatures were 55oC for all primers. Primers used for FoxP3 were as follows FoxP3F:GAAAACACGATTTCCAGAGTTTC; FoxP3R: ATGGCCGAGCGGTGAG; for the CCR6 - CCR6F2: GGCAGTAAAGTGATTCCG; CCR6R2: GCTGTCCTTTGCTCCTGTA; and for CD85j were - CD85jF1: GATCAAGGTACCAATCTCAA; CD85jR1: TCAAGGCCTGCTGAGCCACCGAGCT. Beta-actin was used as the housekeeping gene to further normalize for reverse transcription and PCR efficiencies; although the variability was minimal (not shown). A longer PCR product encompassing this target sequence was used as standard.

**Data analysis**

All results are expressed as mean±SEM or median/quartiles depending on the distribution of samples. Statistical comparisons were made with the use of Student's t tests, ANOVA or non-parametric tests (depending on the distribution of samples). Non-parametric Spearman's correlation test was used to determine relationships between gene expression and ROS production. A value of $P < 0.05$ was considered significant.
RESULTS

High oxidative stress in oral cancer

All studied samples of oral cancer, showed significant production of superoxide anion as measured by lucigenin enhanced chemiluminescence (Fig. 1A). Interestingly, no relationship was observed to the location of changes or the stage of cancer (assessed by TNM) with superoxide production (data not shown).

Mechanisms of oxidative stress in oral squamous cell carcinoma

In order to investigate the sources of superoxide anion in oral squamous cell carcinoma, consecutive segments of tumor from individual subjects were incubated with oxidase inhibitors L-NAME (300 µM) and Apocynin (100 µM). Apocynin, the NADPH oxidase inhibitor showed nearly complete (70 - 90%) inhibition of superoxide production detected by lucigenin-enhanced chemiluminescence (Fig. 1A). Additional inhibition was exerted by L-NAME, the nitric oxide synthase inhibitor (Fig. 1A).

These data were confirmed by the measurements of NADPH oxidase activity in these tissues, which was observed in all samples and was virtually abolished by pre-treatment with p47 phosphorylation inhibitor apocynin (Fig. 1B). This additionally indicates that the NADPH stimulated activity in oral cancer samples is most likely related to p47phox utilizing oxidase - nox2 (and maybe nox1).

![Graph A](image1.png)

![Graph B](image2.png)

*Fig. 1. Characteristics of the basal and NADPH-stimulated superoxide anion production in oral squamous cell carcinoma.* Superoxide production was measured using lucigenin enhanced chemiluminescence (LGCL; 5 µM) in absence (baseline; panel A) and in the presence of 100uM NADPH (NADPH oxidase; Panel B). The effects of pre-incubation with L-NAME (300 µM) and apocynin (100 µM) were assessed. Points indicate individual values; lines indicate mean values. Significance was calculated using student t test for dependent samples.
Nitric oxide synthase dysfunction in oral cancer

Nitric oxide synthase expression has been widely studied in various types of cancer. Inducible NOS is considered to be a major source. However Surprisingly, L-NAME, the nitric oxide inhibitor showed significant inhibitory effect on tumor superoxide production, indicating that nitric oxide synthase may be an additional source of superoxide production in oral cancer (Fig. 1A). The effects of L-NAME were much less pronounced and variable than of Apocynin. In 2/10 samples L-NAME caused an increase of superoxide detection, indicating than NOS in these samples was a net producer of nitric oxide rather than superoxide. Interestingly, in control, non-cancer samples of human mucosa and epithelium L-NAME caused increase rather than decrease of LGCL indicating appropriate function of NOS. In 8/10 cancer samples L-NAME caused variable level of decrease in superoxide production, indicating that NOS in oral cancer is dysfunctional and becomes a net superoxide rather than nitric oxide source. (Fig. 2A). L-NAME inhabitable fraction of basal superoxide production was correlated to NADPH oxidase activity in the same cancer sample indicating a relationship between the two.

Expression of selected immune markers in oral cancer

We next assessed the expression of 3 adaptive immunity related markers, expression of which has been postulated to be related to oxidative stress. These

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**Fig. 2. Nitric oxide synthase dysfunction in oral squamous cell cancer. Panel A.** L-NAME inhabitable superoxide production (L-NAME inhabitable ROS) was measured using LGCL in the absence and presence of NOS inhibition by L-NAME in epithelia/mucosa of control subjects (n=3) and samples or oral cancer (n=10). Data are presented as mean +/- SEM; *p<0.01. **Panel B.** Relationship between NADPH oxidase activity in cancer samples and NOS dysfunction. Linear regression statistics: R²=0.5; p<0.05; n=10.
included FoxP3 (transcription factor involved in T regulatory cell function; Fig. 3A), CCR6 (a marker of effector T cells; Fig. 3B) and CD85j (a marker of T cell senescence; Fig. 3C). We observed that FoxP3 levels in oral cancer samples was significantly diminished when compared to control tissues (30 ± 9 vs. 55.1 ± 8.9 AU mRNA/ beta actin mRNA; p<0.05) and so was the expression of T cell senescence marker CD85j (3.1 ± 1.2 vs. 6.2 ± 2.1 AU; p<0.05). In contrast CCR6 levels were increased in tumor tissue (26.5 ± 9.7 vs. 4.1 ± 0.5 AU; p<0.0001).

Fig. 3. Relationships between selected immune parameters of oral squamous cell cancer and tissue superoxide production by NADPH oxidase (left panels) and NOS (right panels). mRNA expression of FoxP3 (Panel A), CCR6 (Panel B) and CD85j (Panel C) was measured by quantitative real-time PCR. Superoxide production was determined by lucigenin-enhanced chemiluminescence (LGCL; 5 μM). Correlations were analyzed by Spearman correlation statistics.
of these changes indicate the presence of significant T cell dependent immune response in oral squamous carcinoma. We next investigated the relationships between the expression of these immune markers and NADPH-induced superoxide anion production (NADPH oxidase activity) and L-NAME inhibitable superoxide production (NOS superoxide production) from tissue samples.

Relationships between NADPH oxidase activity and immune markers of oral cancer

We observed inverse relationships between the expression of mRNA for analyzed markers of adaptive immunity and NADPH oxidase activity, however it reached statistical significance only in relation to CD85j - a marker of T cell senescence (Fig. 3; left panels).

Relationships between NADPH oxidase activity and immune markers of oral cancer

Similarly to total NADPH oxidase activity, the L-NAME inhibitable superoxide production, being a marker of NOS dysfunction was significantly inversely correlated to the markers of T cell senescence (CD85j) while the other markers did not reach statistical significance (Fig. 3; right panels).

DISCUSSION

Reactive oxygen species appear to play an important role in the regulation of cancer cell growth, differentiation, and angiogenesis (21, 22). However they also possess some tumor preventive effects in a variety of carcinogenesis models. Dual role of ROS in cancer may also be related to differential regulation of redox dependent genes depending on current status of the cell, particularly NFkB (23). Overall, ROS, and in particular superoxide anion play a signaling role in carcinogenesis acting as a metabolic messenger thus influence mainly the promotion and progression stages of experimental carcinogenesis (21, 22). ROS are believed to be important mediators in mutagenesis and carcinogenesis of the upper aerodigestive tract (head and neck), which includes the oral cavity. Additionally to direct changes within cancer cells reactive oxygen species may modify immune responses, thus balance between nitric oxide and superoxide within the tissue may actually translate into the regulation of immune cell recruitment and immune response to cancer (3, 4). Dysregulation of the balance between nitric oxide and superoxide may also affect the role of stem cells in the development of cancer and tissue repair (24). Importantly, such in-balance can be corrected by some therapies as well as non-pharmacological approaches such as exercise or appropriate nutrition (25 - 28).

In the present study we confirm earlier findings in other models, that superoxide anion production is increased in oral squamous carcinoma. We extend these findings by further characterizing superoxide production in oral cancers. We
find that while NADPH oxidase remains the major source of ROS, and particularly superoxide anion, dysfunctional nitric oxide synthase plays an important role as well. This is an important finding as dysfunctional nitric oxide synthase serves often opposite cellular functions than the properly functioning enzyme.

In the present study we did not investigate the mRNA expression of NADPH oxidase subunits or nitric oxide synthases in cancer samples as this has been demonstrated in other studies before (29, 30). Moreover, it is important to note, that expression of these enzymes is not directly connected to their activity as superoxide producing activity of both NADPH oxidases and iNOS or eNOS is tightly regulated through phosphorylation (as is in the case of NADPH oxides, which we also demonstrate using apocynoin - p47phox phosphorylation inhibitor) and availability of substrates and co-factors such as tetrahydrobiopterin (in the case of NOS) (12).

The second issue that our study addressed was the potential relationship between the expression of certain immune related genes (such as FoxP3, CCR6 and CD85j) in oral cancer and its relationship to tumor superoxide generation by NADPH oxidase and by dysfunctional NOS.

Our study opens several further questions that need to be addressed. In particular, it is important to understand which form of nitric oxide synthase could be involved. Based on data published so far (31), it can be speculated that iNOS dysfunction may play an important role here. However expression of other isoforms of NOS have been demonstrated in various models of cancers (31).

It is important to note that oral cancers show particular characteristics, which may be important for the interpretation of the results. Over 90% of oral cancers are squamous cell carcinoma (SCC) where the neoplastic process develops in squamous epithelium. SCC of the head and neck is the seventh most frequent cause of cancer death worldwide (1). The survival rate is among the lowest of the major cancers and has not improved significantly in the past two decades (1). Oral cancer is well known to be more common in men than women. There are several publications which have suggested that the prevalence of this disease is increasing more in women than in men in western countries due to increased smoking and alcohol use (1). The population of subjects from whom the samples were obtained in the present study is typical for oral cancer patients.

Several sources of superoxide anion have been discussed in cancer (21, 22). Mitochondrial electron transport is most well studied followed by NADPH oxidases, peroxisomal reactions, cyclooxygenases and other arachidonic acid metabolism. Interestingly, elevated ROS levels have been associated with the malignant phenotype (21, 22). Importantly, overexpression of NADPH oxidases induces tumor formation in mice (32). The NADPH oxidase is a multi-subunit complex that generates superoxide in the O2 reduction process using electrons supplied by NADPH. NADPH oxidase has been first identified in phagocytes - neutrophils and macrophages - and generates superoxide within the oxidative burst used to kill bacteria and fungi (12, 13, 33). In phagocytes, the NADPH oxidase consist of two membrane proteins, gp91phox and p22phox, that bind a flavin
adenine nucleotide (FAD) and three cytosolic proteins (p47^{phox}, p67^{phox}, p40). Also essential are cytosolic components including the small GTPases (Rac1 and Rac2). The non-phagocytic NADPH oxidase consists of the same components but with a delayed time course for activation and a lower level of activity (e.g. endothelial cells, vascular smooth muscle cells). Some studies suggest distinct differences in amino acids sequence between the central subunits (gp91^{phox} and p22^{phox}) thus different gp91^{phox} homologues are called Nox-1 (Mox-1), Nox-2, Nox3, Nox-4, Nox-5 (12). The presence of NADPH oxidases in different types of cells is important because ROS play autocrine as well as paracrine functions (34).

Various cancer cells and cell lines were found to possess functional NADPH oxidase Nox2 as well as other homologues such as Nox1 and Nox5. For example, the involvement of NADPH oxidases in ROS production were proved by studies with pancreatic carcinoma cells, hepatoma cells, colic adenocarcinoma cells, prostate cancer cells and melanoma cells and many others (21, 22).

The sources of cellular ROS production by human head and neck carcinoma have not been yet fully determined. Recently the increased level of p67^{phox} protein was observed in synthetic retinoid - stimulated ROS production by head and neck SCC, although blocking its expression did not result in significant change in response (12). However, little is known about expression and activity of NADPH oxidases and their functions in the oral SCC. Our findings not only clearly indicate NADPH oxidase activity in oral cancer tissues but may also indicate that it is an NADPH oxidase utilizing p47^{phox}, as its activity is virtually abolished by apocynin, the p47^{phox} phosphorylation inhibitor. These could include predominantly Nox2, and to the lesser extent Nox1 (it uses homologues of p47^{phox}, which could be sensitive to apocynin). We did not study the expression of individual NADPH oxidase homologues in the present study, and currently there are no sufficiently specific inhibitors of individual Nox enzymes to investigate the contribution of individual homologues.

While the importance of superoxide in mediating cellular growth and proliferation has been studied in large extent, relatively little is known regarding the relationships between ROS production or superoxide anion generation within the cancer tissues and immune responses to the tumor (35). Thus in the present study we looked at the relationships between superoxide production in tumor tissues and mRNA expression of certain T cell and NK cell specific genes, as indicators of immune reaction and its characteristics.

Cancer tissues constantly protect themselves from host defense, which can be mediated by attracting regulatory T regulatory cells (T regs) (36) that suppress immunological response and enable to escape from the host immune surveillance (37, 38). They can produce immunosuppressive cytokines, such as CCL22, IL-10 and prevent the production of these cytokines that recruits T cells to tumors. Finally they can induce immune senescence, increasing the threshold of activation of cytotoxic cells such as CD8+ or NK cells (38). In our study, we investigated in part these aspects of anti-tumor immunity in the oral cancers.
There is very little known about the impact of the adaptive immunity for the development and the progress of the malignant lesions in the oral cavity (38). We examined the CCR6 expression which is the mucus and skin homing receptor that mostly occurs on dendritic cells but on T cells as well (36). The level of the CCR6 expression in the cancer tissue helps to judge on the magnitude of the immune system reaction to the cancer. It has also been shown recently to identify certain metastatic properties of SCC of head and neck (36). If the production of the CCL 20, which is CCR6 ligand, in the cancer area is suppressed by the tumor, the recruitment of the DC and T cells to fight the cancer should be low. However we found that CCR6 levels were significantly higher in oral SCC cancer tissues then in control tissues. Interestingly NADPH oxidase activity has shown a trend toward the inverse correlation with CCR6 mRNA in tumor. Although it did not reach statistical significance, it must be noted that number of samples available to us in a present study allow us to draw only preliminary conclusions. Our observation could indicate that CCR6+ cells are less attracted to tumors with high oxidative stress, which could be related to lowering of the CCR6 ligand (CCL20) in cancer tissues in relation to oxidative stress. If the tumor is not capable to diminish CCL 20 production (low NADPH oxidase activity samples), the answer of the immune system to the malignant tissue is much stronger. In order to characterize potential mechanisms by which T cells could be inhibited in oral cancer we studied the expression of the Foxp3 gene as a marker of T regulatory cell infiltration of the cancer tissue (36). Surprisingly its expression was lower in cancer samples than in control samples, suggesting that in oral cancer this may not be a major mechanism diminishing immunity to cancer. Similarly to CCR6 there was a trend towards the inverse relationship between FoxP3 mRNA and NADPH oxidase activity, however it did not reach statistical significance. Finally, we investigated the CD85j mRNA expression in oral SCC. CD85j is the immunoglobulin-like MHC class I inhibitory receptor which is characteristic for NK cells and is abundant on CD8 cells as well (39, 40). It is present only in a small percentage of CD4 cells. CD85j is thought to participate in tolerance induction in NK cells or possibly CD8 cells by raising the threshold of their activation. Interestingly CD85j expression was statistically significantly inversely correlated with both NADPH oxidase activity and NOS dependent superoxide production, indicating that the higher superoxide production, the lower CD85j+ cell infiltration of the tumor - thus in line with CCR6+ correlation - tumor is less likely to be subjected to immune mediated killing.

In summary, our study shows that apart from NADPH oxidase, the dysfunctional nitric oxide synthase is responsible for superoxide anion production in these tissues. More importantly our pilot study suggests that the oxidative stress generated by both enzymes may be actually related to the characteristics of the immune response. These observations although preliminary, may be of critical value as modification of oxidative stress in the tumor may actually affect not only the proliferation of cancer cells as previously shown but could also improve the
immune surveillance in cancer. Thus these findings need to be further characterized in larger populations.

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