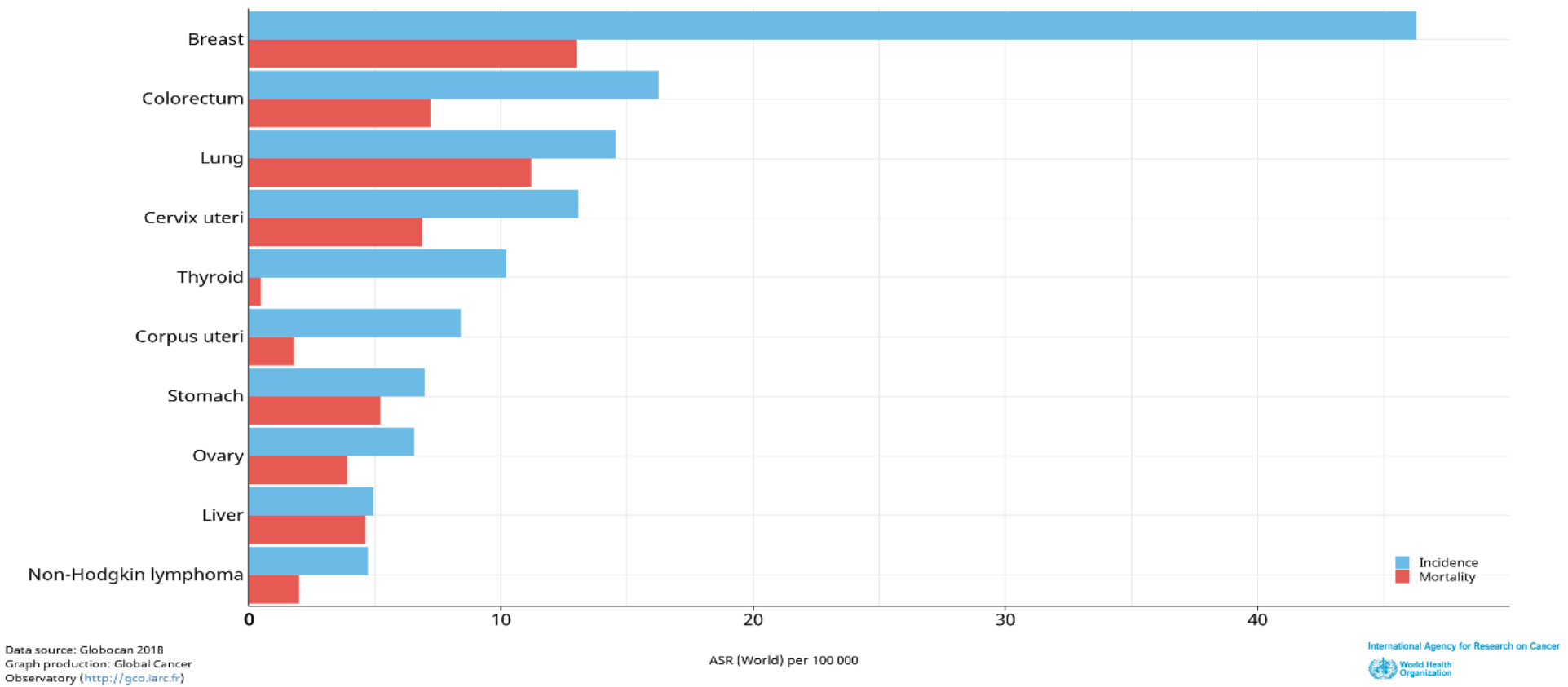
HPV E6 and E7 onco-proteins sensitize Human keratinocytes to oxidative damage

Wilner Martínez-López

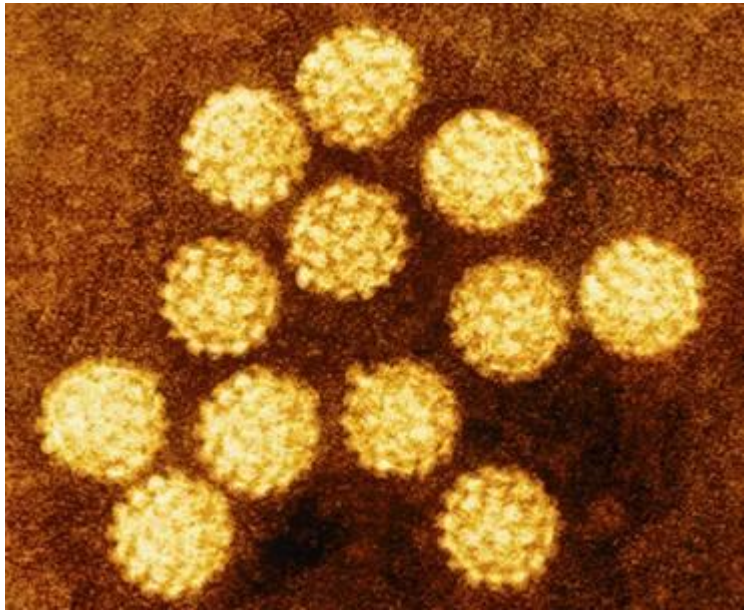
Epigenetics and Genomic Instability Laboratory
Biodosimetry Service
Instituto de Investigaciones Biológicas Clemente Estable (IIBCE)
Montevideo – Uruguay
wilnermartinezlopez@gmail.com

iibce
INSTITUTO DE INVESTIGACIONES BIOLÓGICAS CLEMENTE ESTABLE

Epidemiology of uterine cervix cancer



Human Papillomaviruses (HPV)



Family : *Papillomaviridae*

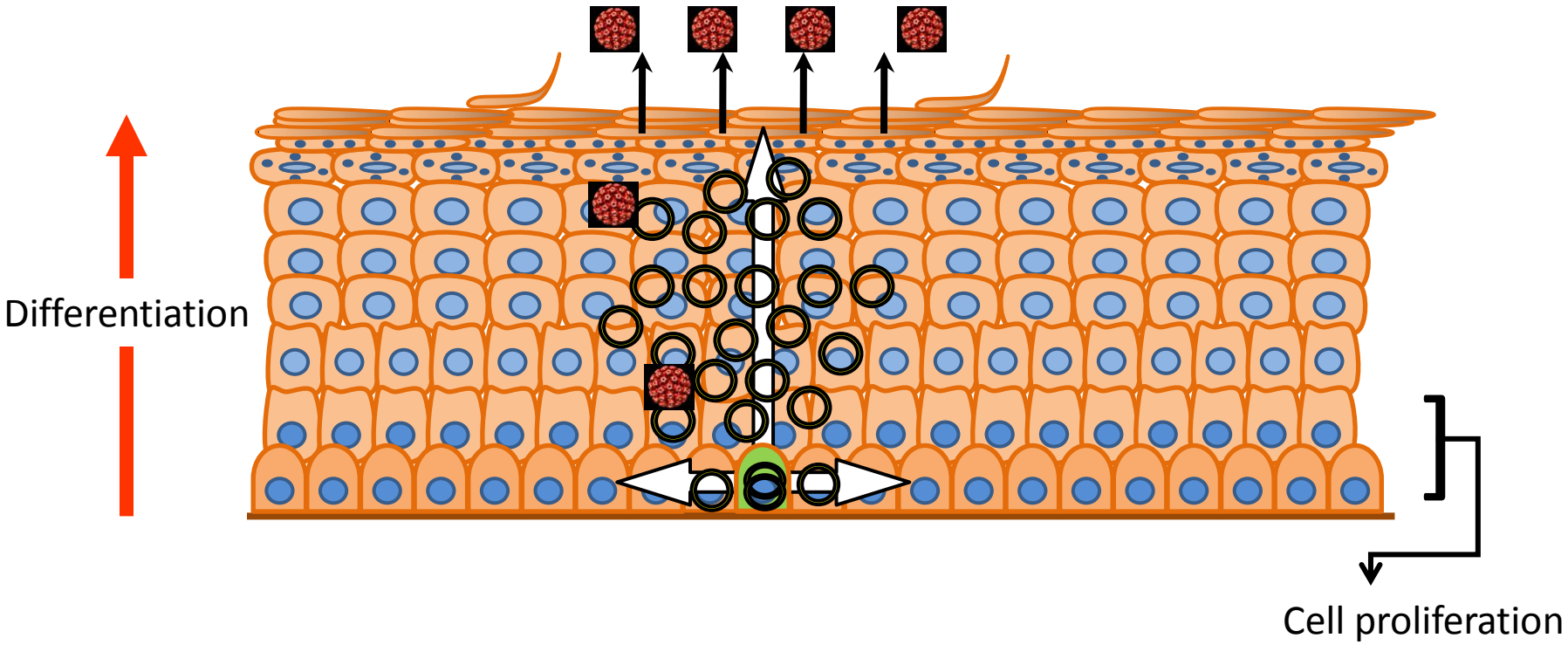
Small non-enveloped virus (~50 nm)

dsDNA circular genome (~8 kpb)

Etiologically associated with:

- Cervical cancer (99,7%)
- Oropharyngeal cancers (up to 70%)
- Vulvar and penile carcinoma (50%)
- Anal carcinoma (up to 80%)
- Laringeal papillomas, common and genital warts (100%)

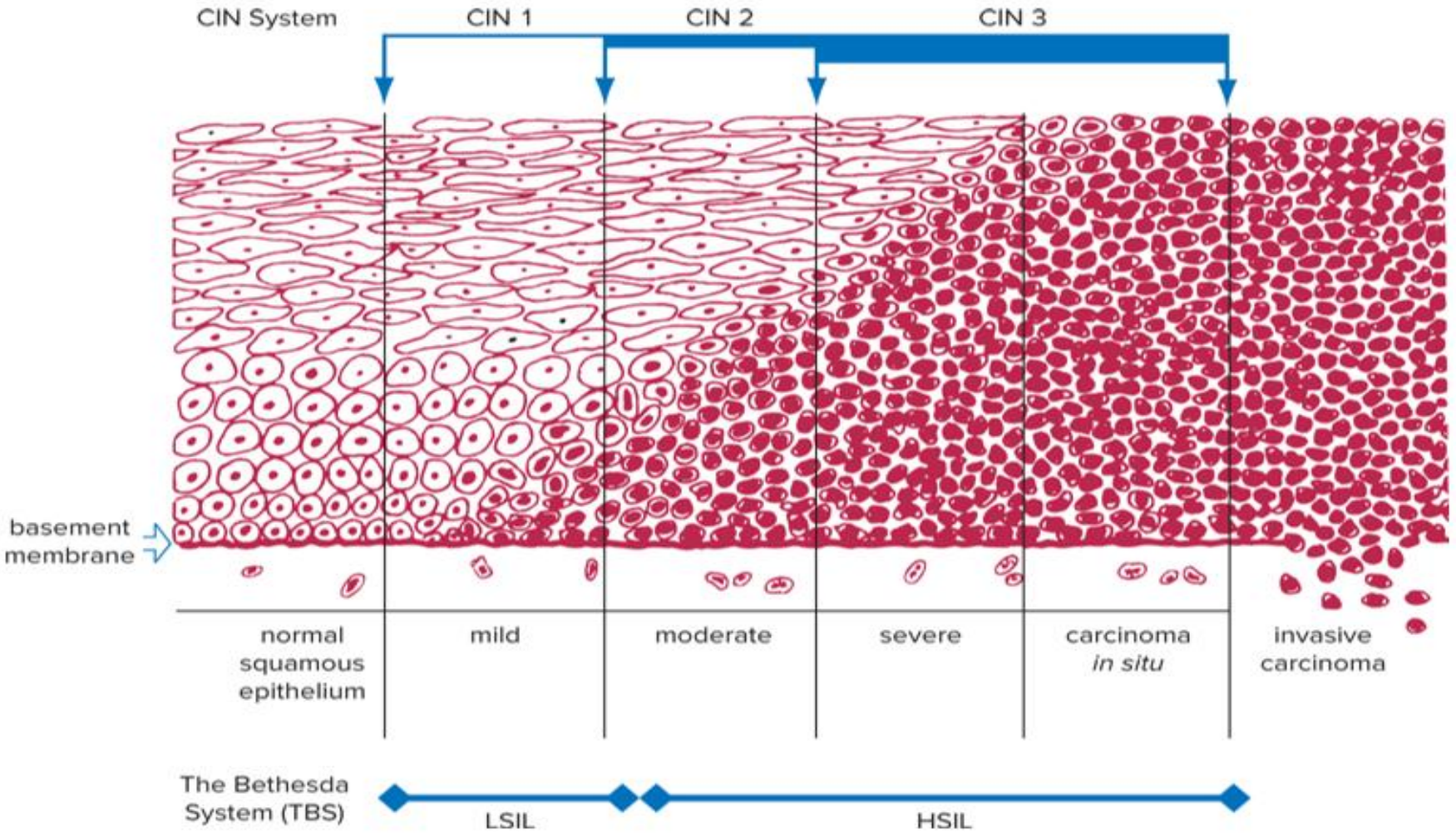
HPV life cycle



Adapted from zur Hausen Nature Reviews Cancer 2, 342-350 (2002)

Progression of epithelial lesions produced by HPV

Cervix Intraepithelial Neoplasia



Low or High Squamous Intraepithelial Lesion

HPV classification

LUDWIG INSTITUTE FOR CANCER RESEARCH

~ 200 HPV TYPES

↓

~ 40 INFECT ANOGENITAL TRACT

↓

LOW RISK
(HPV6, 11, 42, 43, 44)

↓

LOW-GRADE LESIONS
AND GENITAL WARTS

HIGH-RISK
(HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 e 66)

↓

HIGH-GRADE LESIONS
INVASIVE CARCINOMA

High-Risk HPVs express two onco-proteins: E6 and E7

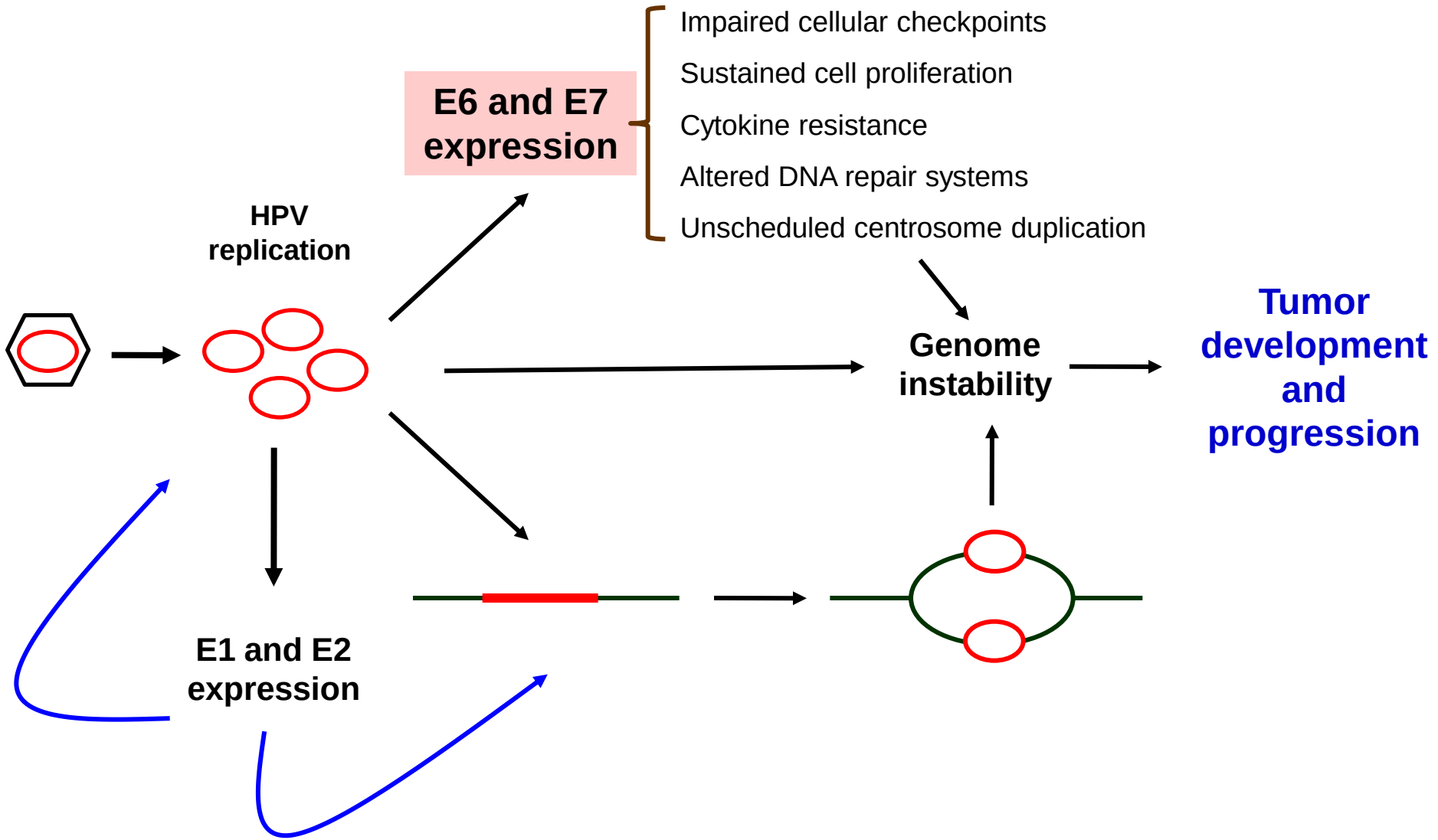
Small proteins with pleiotropic effects :

E6

E7

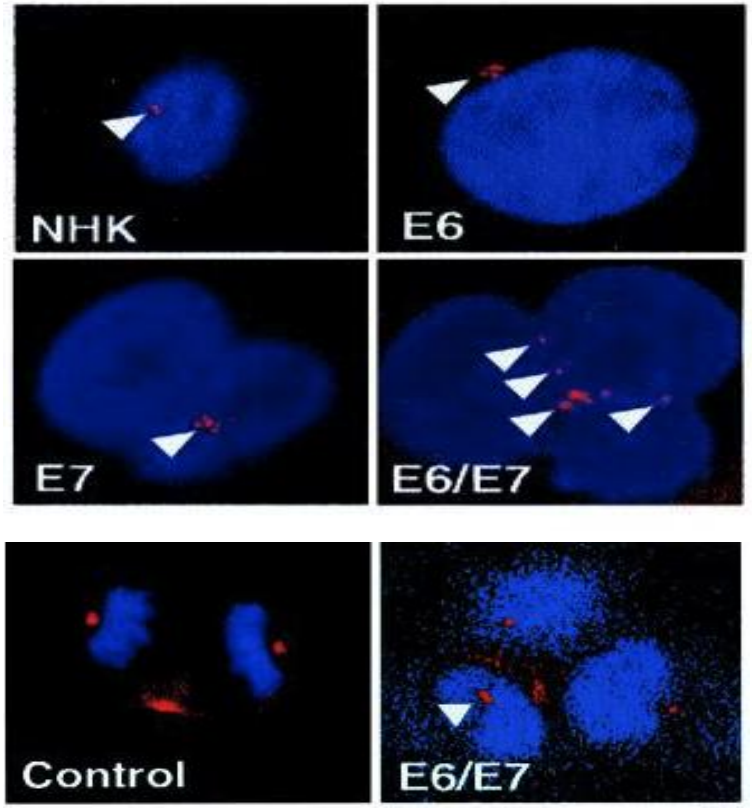
- Collaborate to immortalize human keratinocytes.
- **Promote**:
  - extended cell lifespan
  - unscheduled DNA replication
  - avoid apoptosis
  - evade immune response

HPV and Cancer



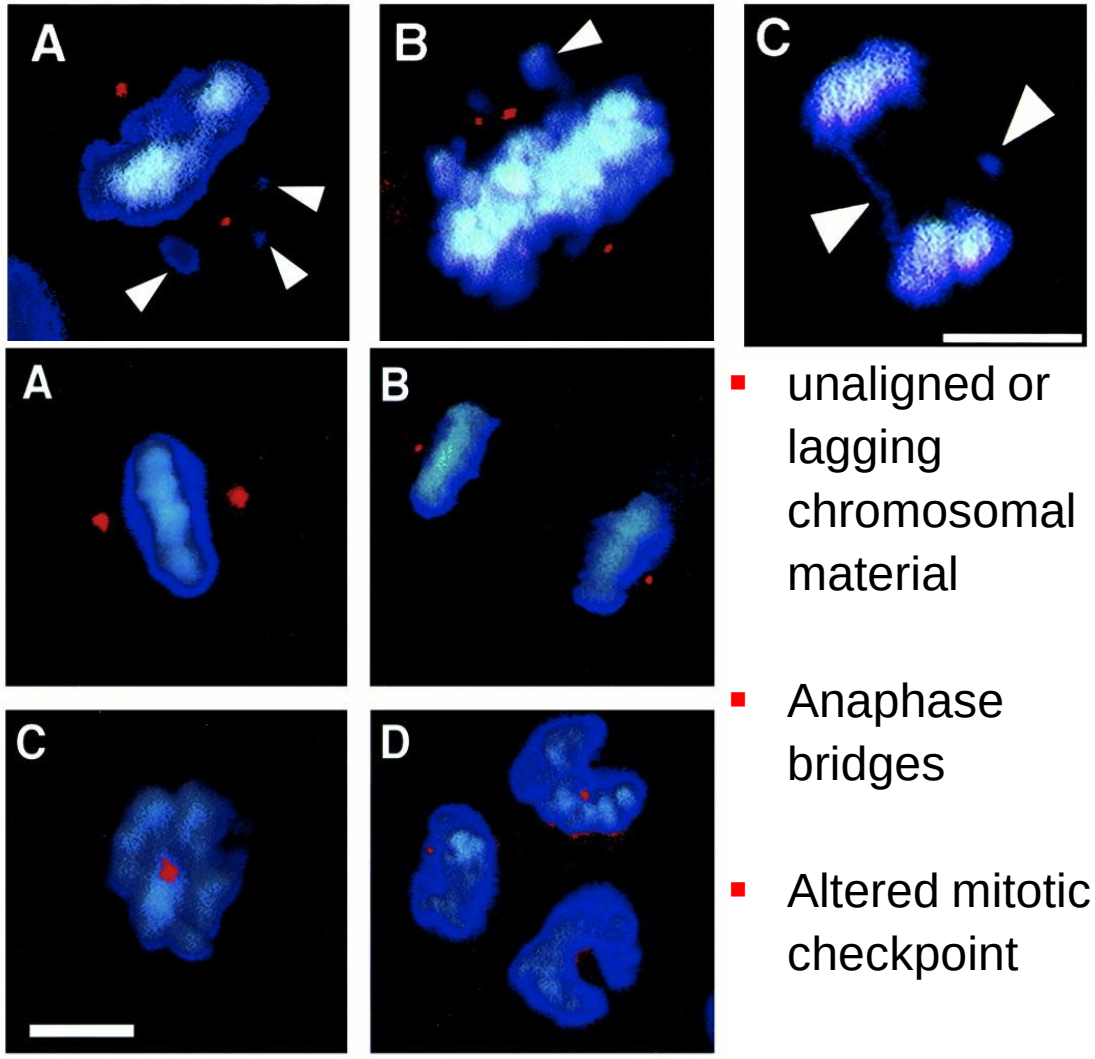
Adapted from Boccardo, 2009, DOI: 10.1159/000290657

Mechanisms of viral-mediated Neoplasia



(Duensing et al., 2000, PNAS, vol. 97:18)

- E6/E7 induce abnormal centrossome numbers.
- multiple spindle poles.



(Duensing e Munger, 2002, *Cancer Res.* 62.7075:82)

- unaligned or lagging chromosomal material
- Anaphase bridges
- Altered mitotic checkpoint

Chronic inflammation and HPV

In spite of the central role of E6 and E7 onco-proteins from HPV in the induction of genomic instability, several epidemiological and molecular studies have demonstrated that the HPV infection and the sustained expression of onco-proteins are not enough to develop cancer. Actually, it has been swhown that additional events between the virus and the host should be required. In this respect, the oxidative microenvironment during chronic inflamation could contribute to the cervical carcinogenesis and tumor progression (Mangino et al., 2016).

The persistance of HPV infection favour the develpment of chronic inflammation as well as the generation of oxidative stress. Chemokines (CXCL1/GROα, CXCL8/IL-8, CXCL5/ENA-78, CXCL12/SDF-1) and proinflammatory cytokines as interleukin(IL)-6 and IL-1α can favour the growth of tumor whereas the treatment with non-steroidal antiinflammatory drugs can reduce cancer incidence and aggressiveness (Williams et al., 2011).

Rationale

A better understanting of the relationship between the sustained expression of E6 and E7 HPV proteins and the senzitation of host cells to DNA damage induced by oxidizing agents, could contribute to develop new strategies on the prevention or treatment of uterine cervix cancer.

Therefore, it has been proposed to study the relationship between oxidative stress and the generation of DNA damage in immortalized human keratinocytes transfected with E6 and E7 HPV16 proteins, with the aim to confirm that accumulation of genetic damage produced by oxidative stress could be in the base of cellular transformation. If it so, it will be intended to test the effect of an antioxidant, such as the ascorbic acid, on the induced genetic damage by oxidative stress on these cells

General objective

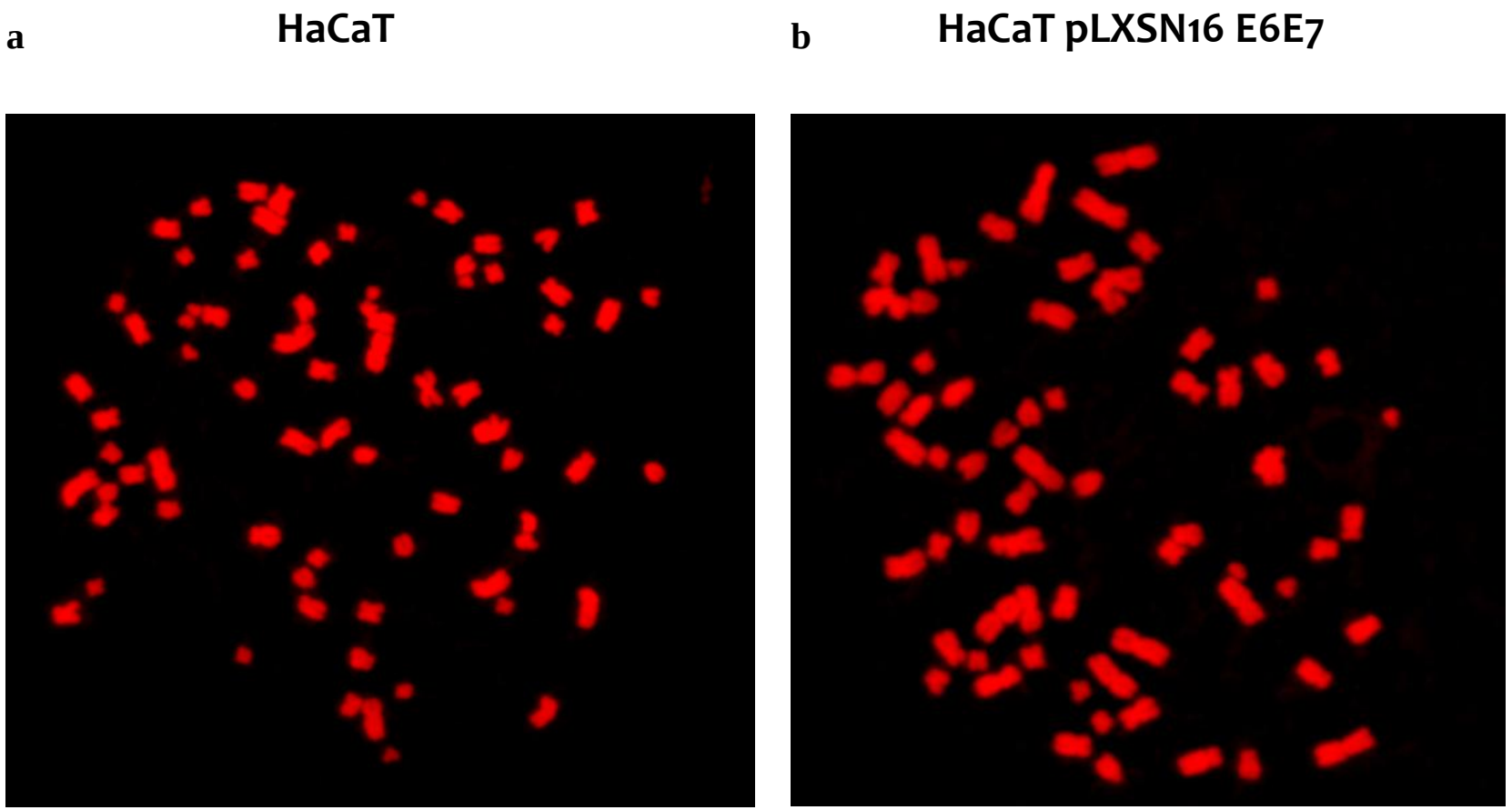
Analyze if the expresión of E6 and E7 onco-proteins from HPV16 sensitize HaCaT cells to oxidative damage induced by hydrogen peroxide

Specific objectives

In HaCaT cells transduced with a lentiviral vector coupled with the E6 and E7 HPV16 onco-proteins kindly provided by Dr. Enrique Boccardo from the Oncovirology Laboratory from USP (Brazil):

- **Evaluate the effect of the oxidizing agent, hydrogen peroxide, on cell viability**
- **Determine the formation of reactive oxygen species after treatment with the oxidizing agent**
- **Quantify the effect of oxidative damage at the chromosomal level after exposure to hydrogen peroxide**
- **Evaluate the primary oxidative damage induced in the DNA by hydrogen peroxide**
- **To study the scavenger effect of ascorbic acid on oxidative stress induced by hydrogen peroxide**

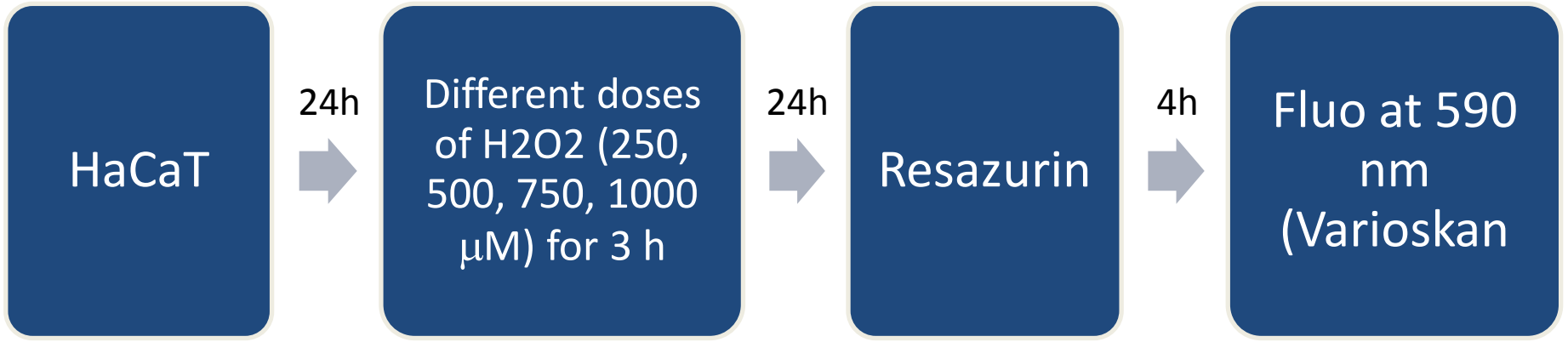
MAIN RESEARCH ACTIVITIES



Cytogenetics analysis of HaCaT and HaCaT pLXSN16 E6E7 cells. a) Metaphase from a HaCaT cell (72 chromosomes); b) Metaphase from a HaCaT keratinocytes lentiviral transduced with E6 and E7 onco-proteins from HPV16 (69 chromosomes). Both metaphases were stained with propidium iodide and captured employing a fluorescence microscope coupled with a CCD camera at 63x magnification.

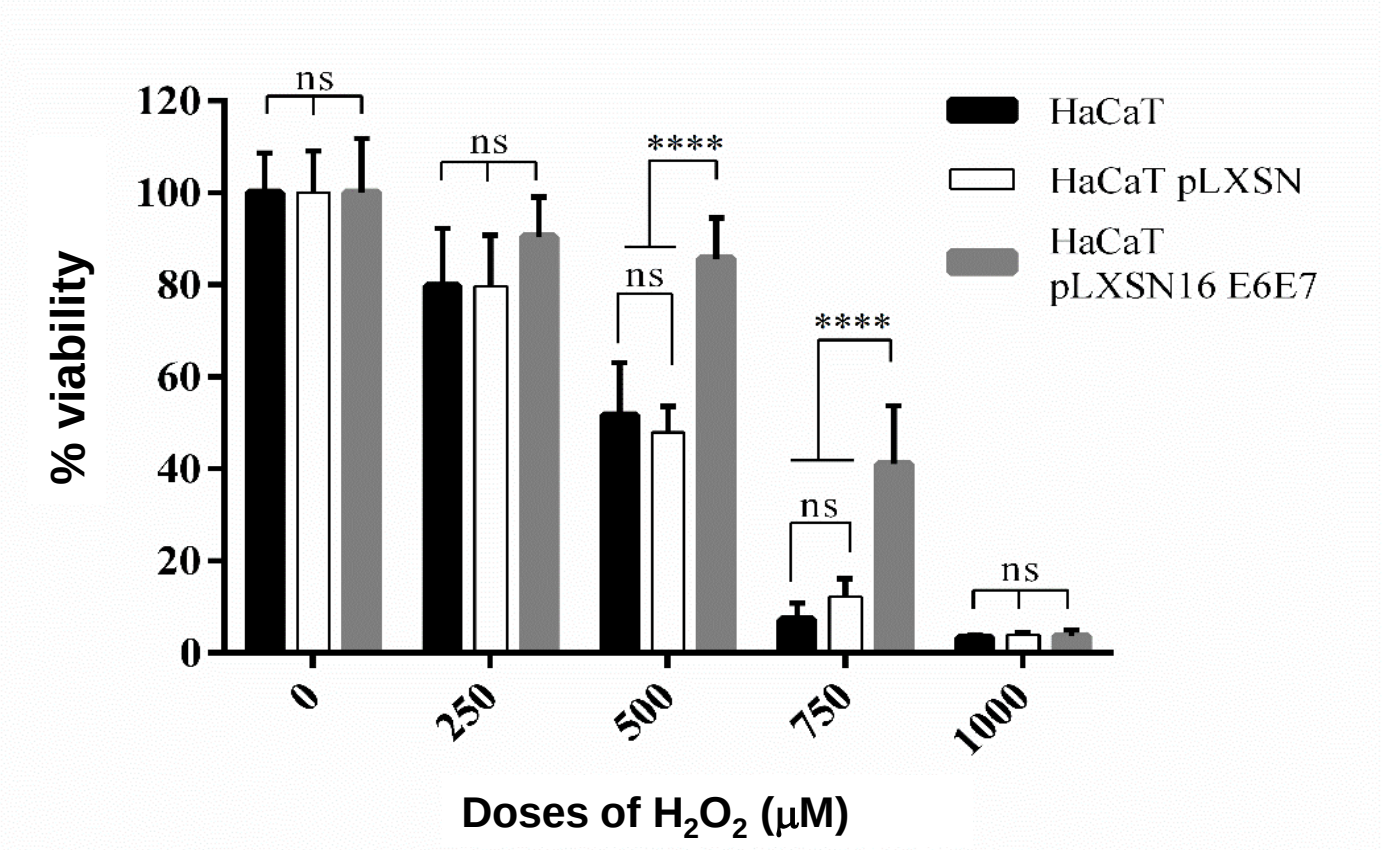
Methodology

Analysis of cell viability by means of Fluorimetric assay Resazurin



Resazurin is a blue dye, itself weakly fluorescent until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin

MAIN RESEARCH ACTIVITIES



Cell viability assay of HaCaT, HaCaT pLXSNØ and HaCaT pLXSN16 E6E7 cells exposed to different concentrations of (250-1000 µM) H_2O_2 for 3 h. The assay was performed in duplicate and six replicas were performed per each experimental point. Statistical analysis: *two-way* ANOVA and Test de Bonferroni, ns: not significant, ****, $p \leq 0.0001$.

Methodology

Analysis of the presence of intracelular ROS

(Fluorimetric Assay employing 2'-7'-diclorodihydrofluorescein diacetate,

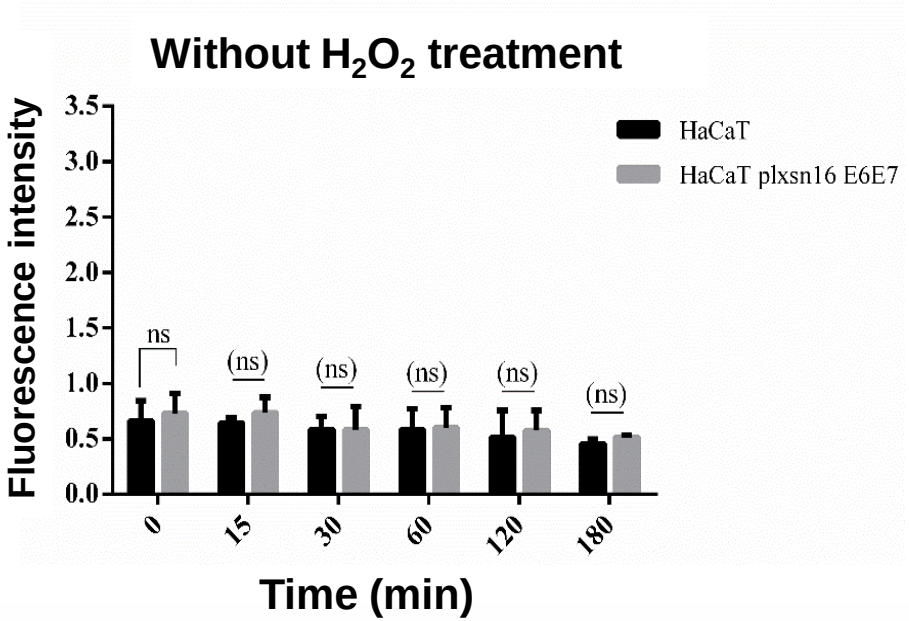
DCFH-DA, Sigma-Aldrich)

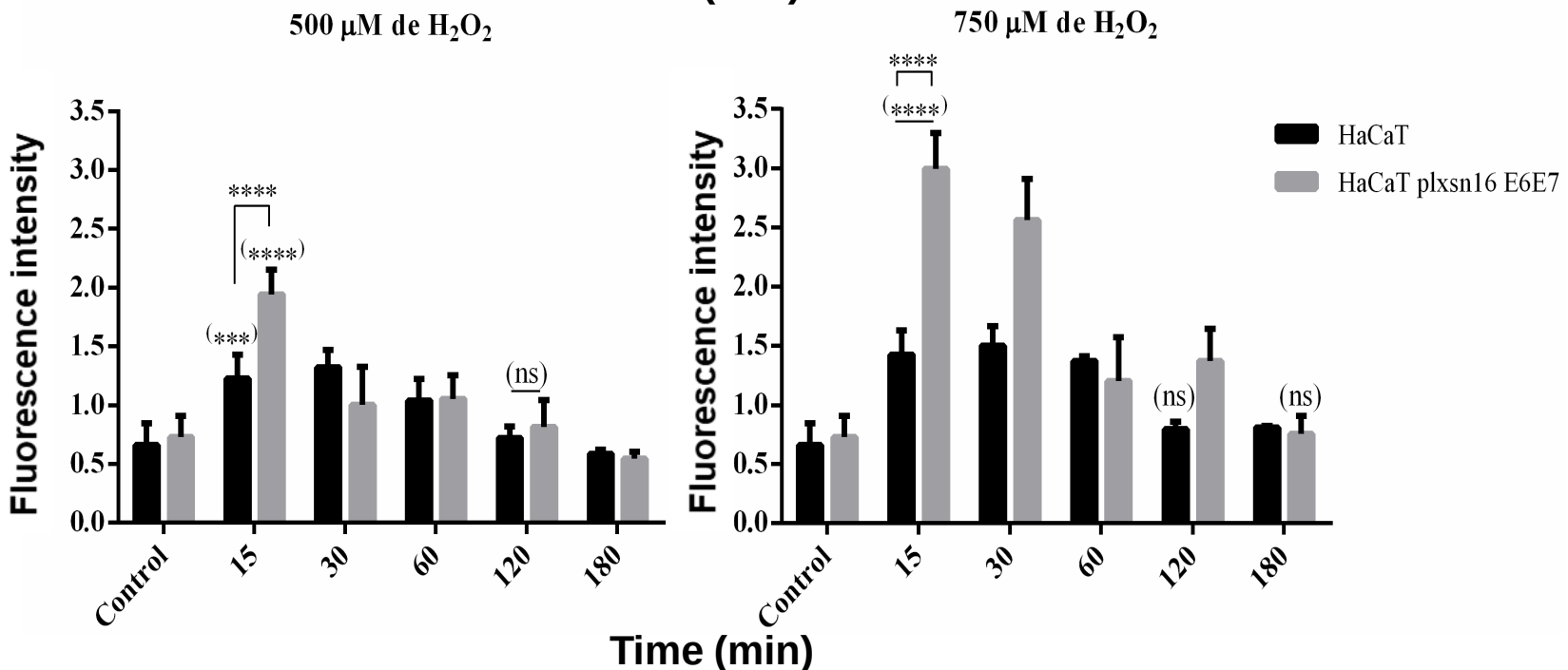
DCFH-DA → (Deacetylated at the Cytoplasm) → **DCFH** → (React with ROS) → **DCF**

MAIN RESEARCH ACTIVITIES

Intracellular ROS activity through DCFH-DA assay

Statistical analysis: *two-way* ANOVA and Test de Bonferroni, ns: no significativo, ***, p ≤ 0.001 y ****, p ≤ 0.0001. Values between () are significance related to controls

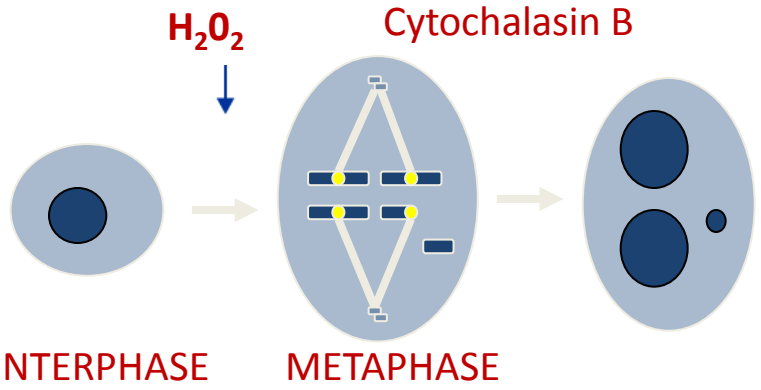




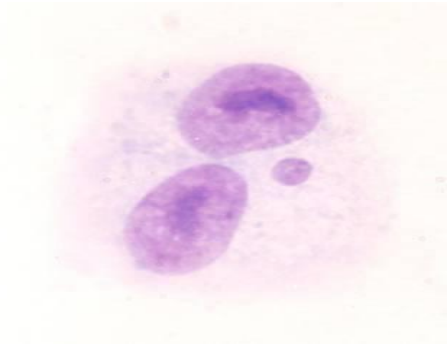
Methodology

Analysis of the accumulated chromosome damage through the study of the frequency of Micronuclei (MN) induced by H_2O_2 employing the CBMN assay.

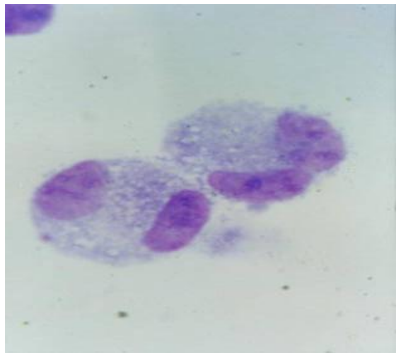
Micronucleos Assay (CBMN)



Ribeiro (2003)



Bi-nucleated cell with a micronucleos



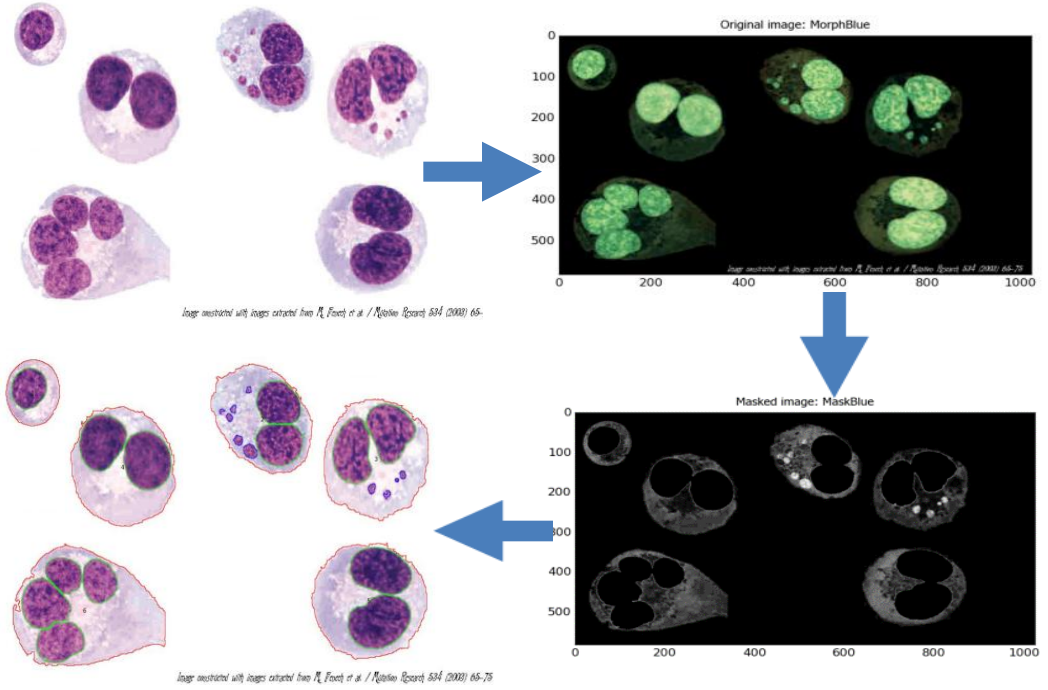
Cells without micronucleos

Automatic analysis of MN

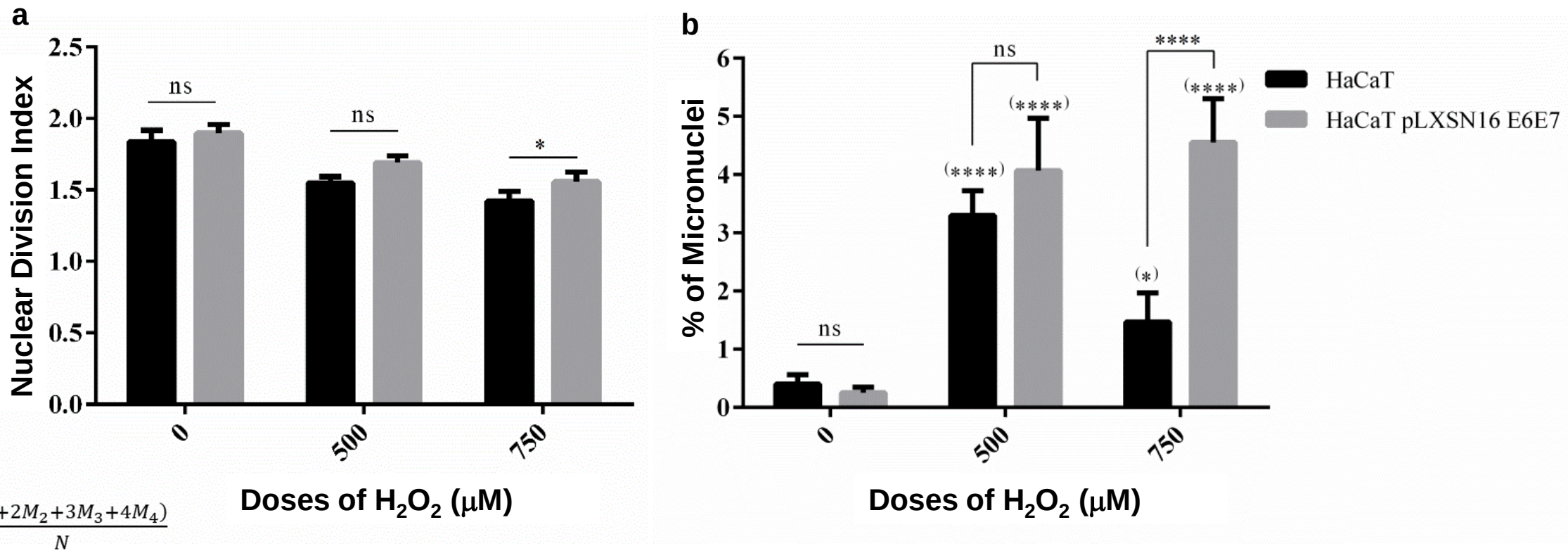
Slide scanning system

MetaPhase Finder

Software Cell Profiler



MAIN RESEARCH ACTIVITIES



$$NDI = \frac{(M_1+2M_2+3M_3+4M_4)}{N}$$

Analysis of chromosome damage by CBMN assay. a) Nuclear Division Index (NDI) estimated in HaCaT and HaCaT pLXSN16 E6E7 treated with 500 or 750 μM H_2O_2 for 3 h; b) Percentage of Micronuclei (MN) in binucleated cells showing accumulated chromosome damage. The assay was performed in duplicate and two replicas were performed per each experimental point. Statistical analysis: *two-way* ANOVA and Test de Bonferroni, ns: not significant, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Values between () are significative in relation to controls.

Methodology

Culture cells

HaCaT or HaCaT pLXSN16 E6E7 treated for 5 min with 750 µM H2O2 and recoverd (0, 15, 30, 60, 120 min)

Cells embedded in agarose (LMP at 37°C) onto glass slides

Lisis (Triton X-100, 2.5 M NaCl)

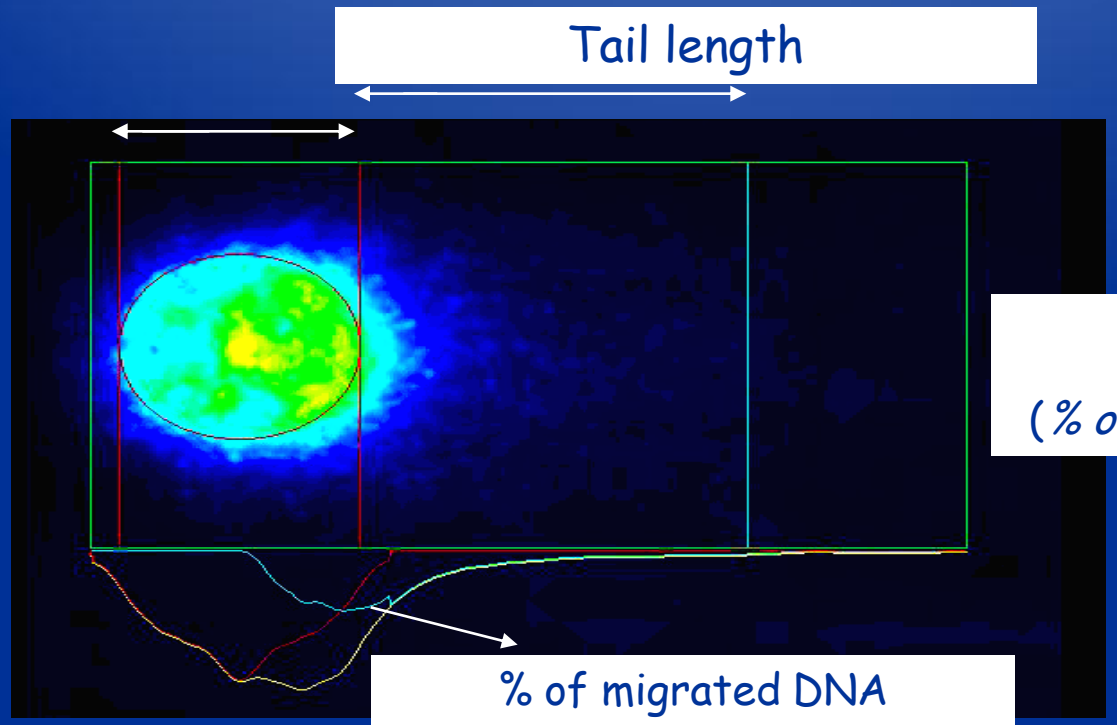
Nucleoid

Alkaline incubation and Electrophoresis

(0.3 M NaOH, 10 mM EDTA)

Quantitation of DNA damage by using Comet Imager (MetaSystems GmbH)

Parameters analyzed

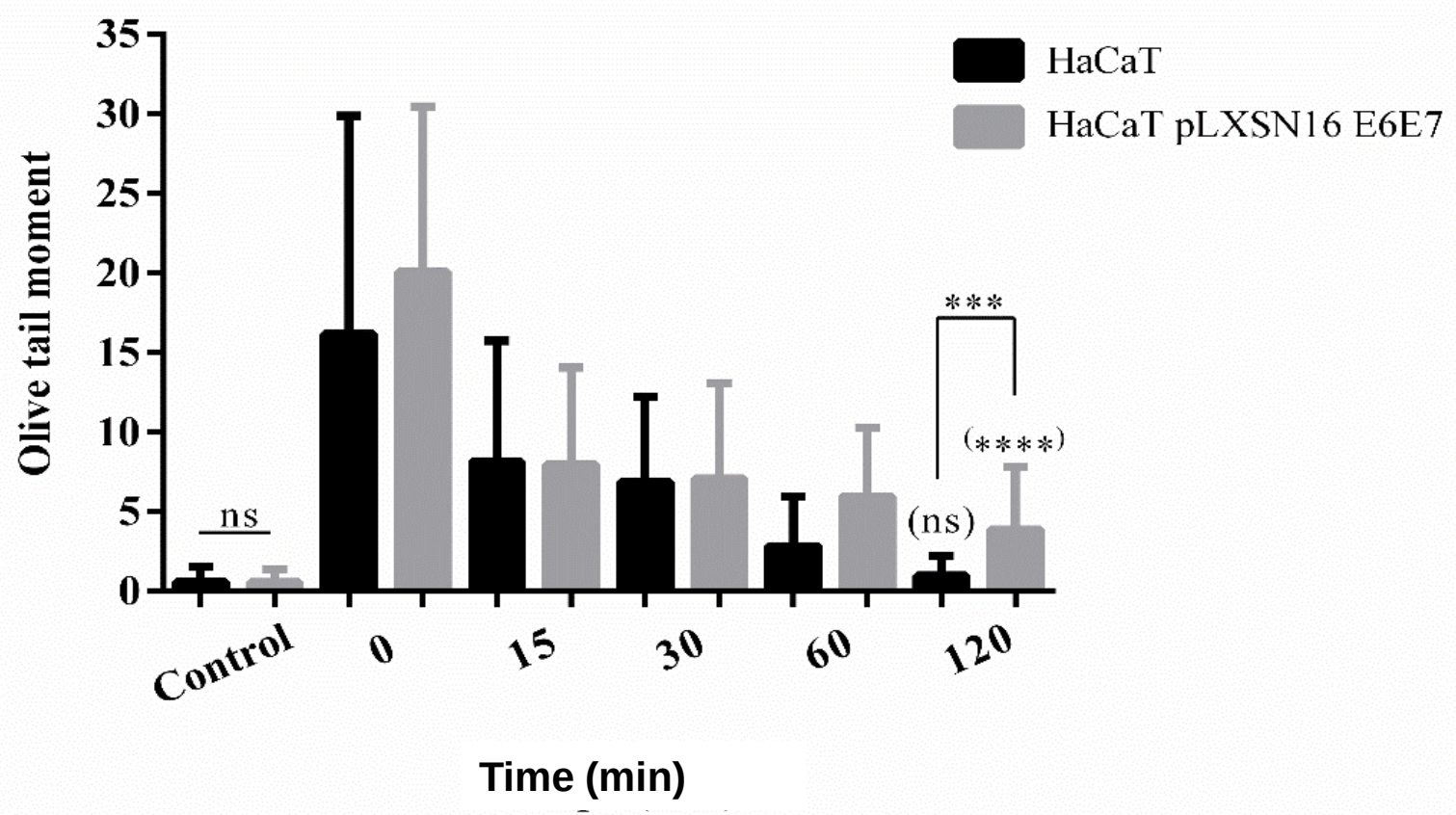


Tail Moment

(*% of DNA in tail x tail length*)

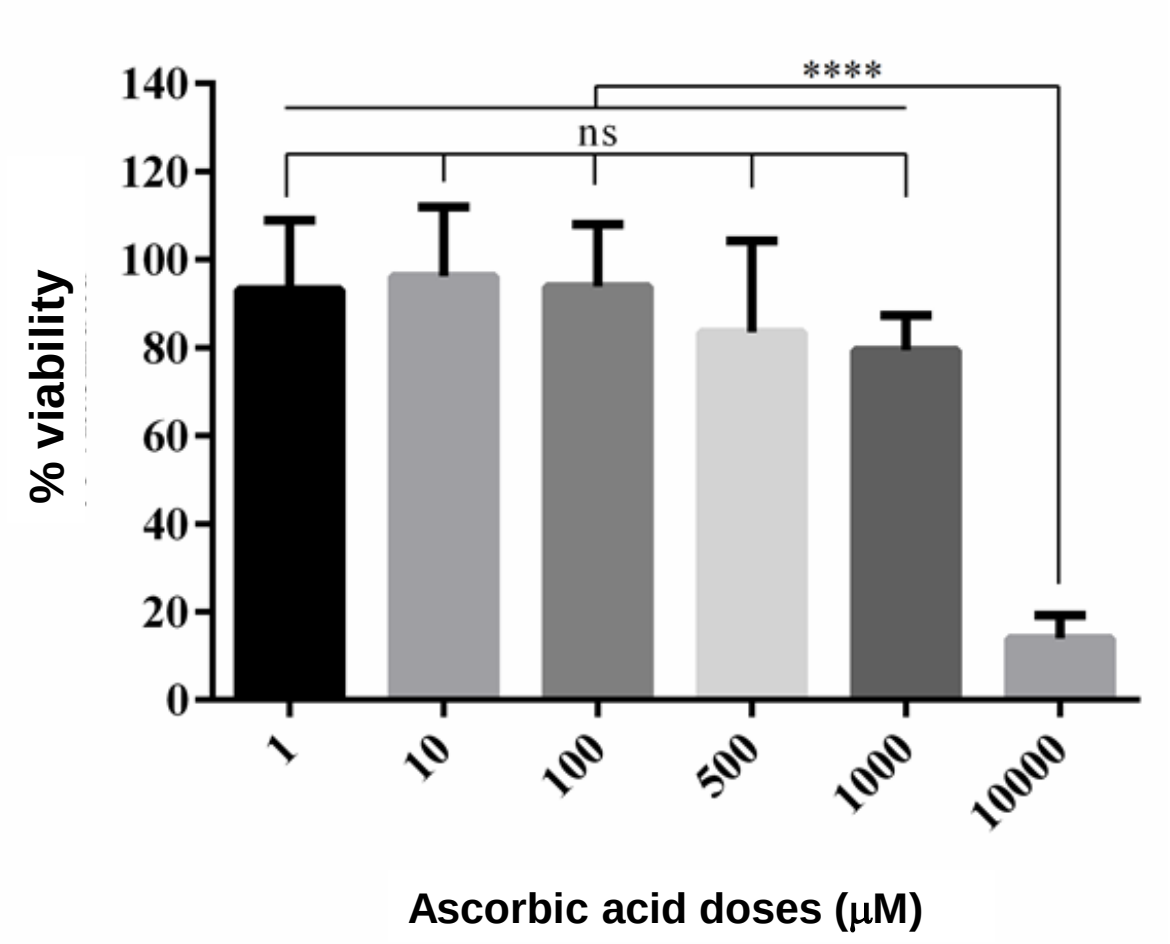
Software CometImager (MetaSystems)

MAIN RESEARCH ACTIVITIES



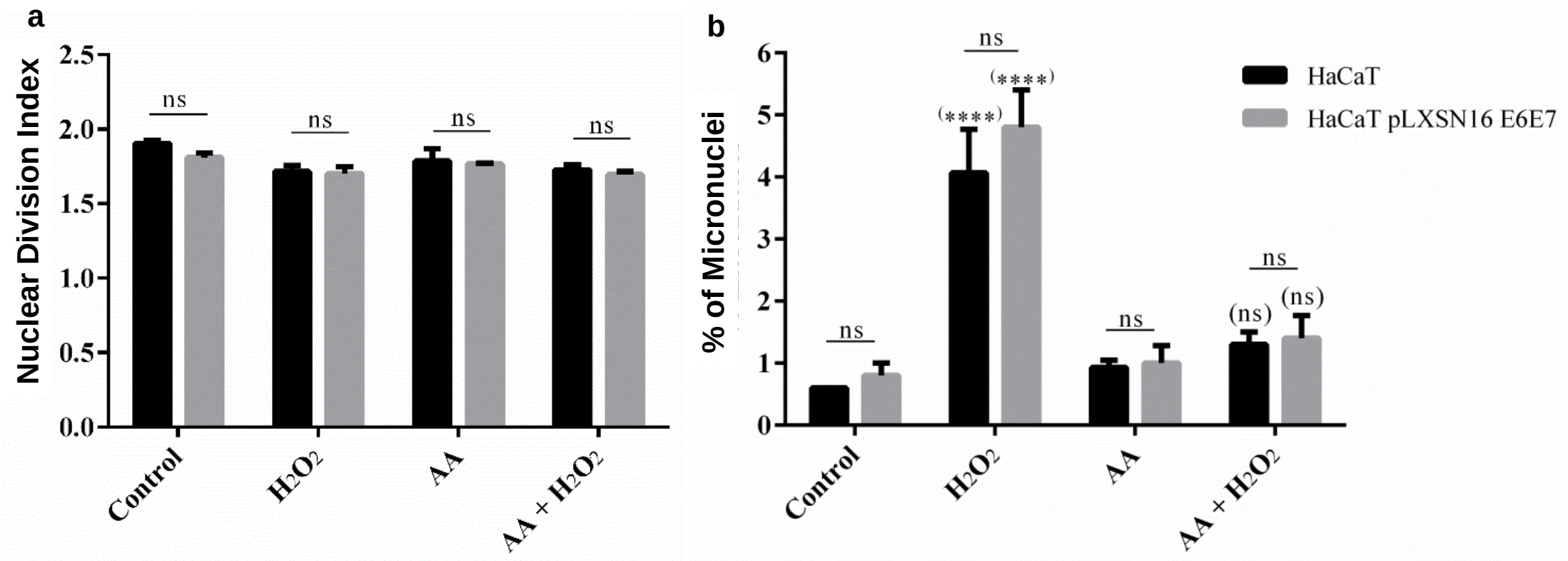
Analysis of primary DNA damge by Comet assay. HaCaT and HaCaT pLXSN16 E6E7 cells were treated with 750 µM H2O2 for 5 minutos and recovered for 0, 15, 30, 60, 120 min. The assay was performed in duplicate and two replicas were performed per each experimental point. Statistical analysis: *two-way* ANOVA and Test de Bonferroni, ns: not significant, ***p ≤ 0.001, ****p ≤ 0.0001. Values between () are significative in relation to controls.

MAIN RESEARCH ACTIVITIES



Cell viability assay of HaCaT cells exposed to different concentrations of ascorbic acid (AA, 1, 10, 100, 500, 1000, 10000 μM) for 24 h. The assay was performed in duplicate and six replicas were performed per each experimental point. Statistical analysis: *two-way* ANOVA and Test de Bonferroni, ns: not significant, ****, $p \leq 0.0001$.

MAIN RESEARCH ACTIVITIES



Analysis of chromosome damage by CBMN assay employing a scavenger. a) Nuclear Division Index (NDI) estimated in HaCaT and HaCaT pLXSN16 E6E7 cells treated with 500 µM H_2O_2 for 3 h and previously exposed to 1 mM of AA for 24 h; b) Percentage of Micronuclei (MN) in binucleated cells showing accumulated chromosome damage. The assay was performed in duplicate and two replicas were performed per each experimental point. Statistical analysis: *two-way* ANOVA and Test de Bonferroni, ns: not significant, ****p ≤ 0.0001. Values between () are significative in relation to controls.

SUMMARY

- The cytogenetic analysis showed that these cell lines do not present spontaneous structural chromosomal rearrangements
- Cell viability diminished when cells were exposed to increased doses of H_2O_2, although transduced cell lines are more resistant than control cells.
- Either cells with or without E6 and E7 onco-proteins showed an inrease in ROS soon after H_2O_2 treatment, although at 750 µM H_2O_2 the transduced cell line significantly increase ROS.
- Transduced cell line is able to accumulate genetic damage evidenced through the analysis of MN, indicating that these cells are more resistant to H_2O_2 as well as they can survive accumulating genetic damage. Events considered important for progression to malignancy.
- Besides, transduced cell line is not able to completely revert genetic damage during the period analyzed by comet assay while control cells were able to remove primary damage at 120 min, which can explain the accumulation of genetic damage showed with the MN analysis.
- Ascorbic acid showed a high efficiency to remove oxidative stress induced by H_2O_2, avoiding the accumulation of genetic damage either in control o transduced cell lines.

CONCLUSIONS

It is well known that the persistance of viral infection leads to a chronic inflamation state, producing an oxidative microenvironment.

In this respect, we have shown in our model, employing a keratinocyte cell line tranduced with E6 and E7 from HPV16, that the presence of these onco-proteins sensitize human keratinocytes to genetic damage induced by H_2O_2, which can be reverted by the addition of a scavenger like ascorbic acid.

BASIC-CLINIC RESEARCH GROUP

Radiotherapy Department (MSP-HPR-Uruguay)

Dra. Mariela Dalla Rosa
Dra. Olga Lillo
Dr. Alejandro De Rosa
Dra. Adriana Castaño
Dr. Sergio Aguiar
Dr. Álvaro Ronco
Prof. Aldo Quarneti

BIODOSIMETRY SERVICE (IIBCE-Uruguay)

Lic. Silvina Acosta
Mag. Diego Alem
Tec. Mario Lalinde
Dr. Maria Vittoria Di Tomaso
Prof. Wilner Martínez-López

USP (BRAZIL)

Prof. Enrique Boccardo (USP)

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MUCHAS GRACIAS !!!