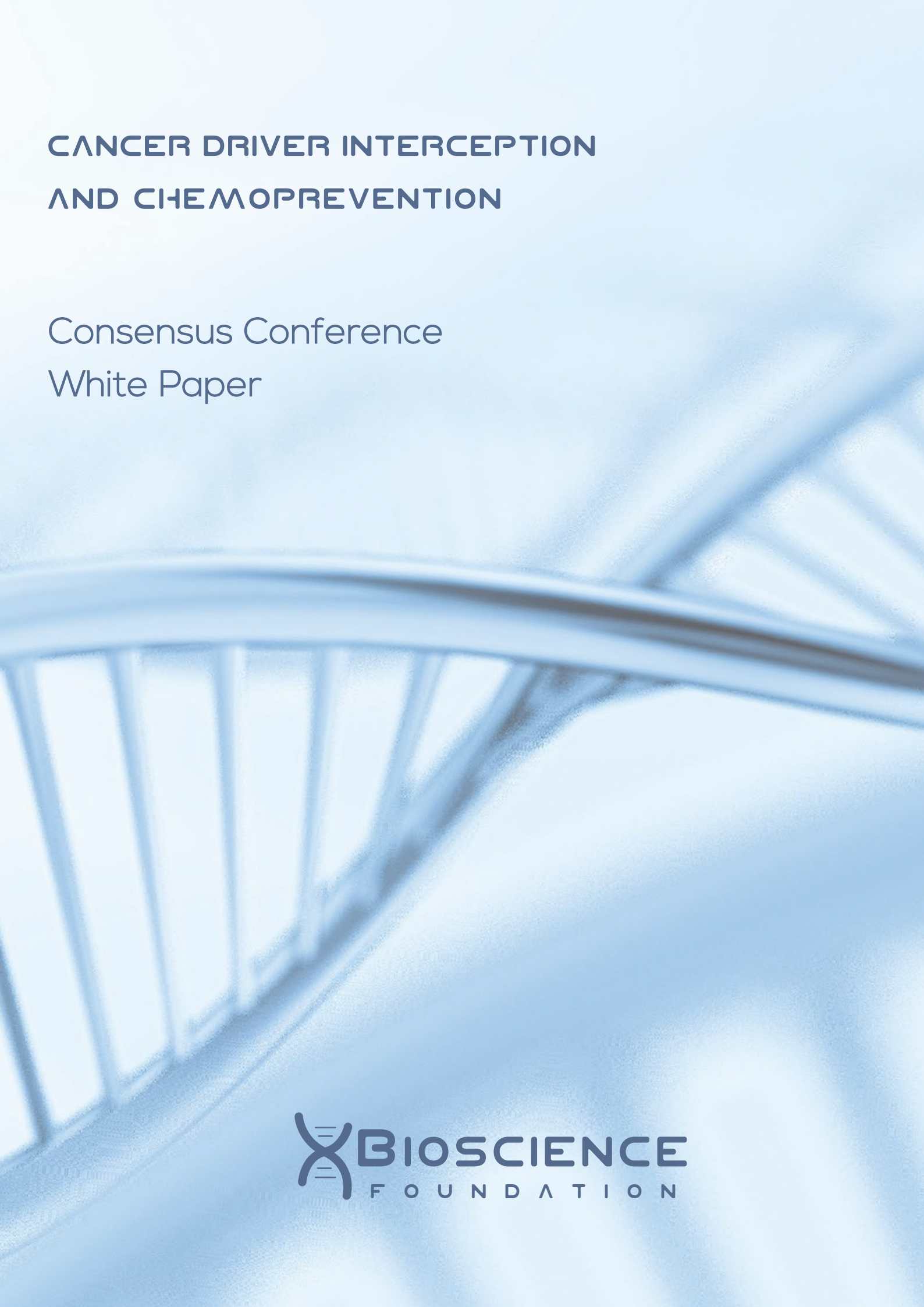
CANCER DRIVER INTERCEPTION AND CHEMOPREVENTION

Consensus Conference
White Paper



BIOSCIENCE FOUNDATION

CANCER DRIVER INTERCEPTION AND CHEMOPREVENTION

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White Paper

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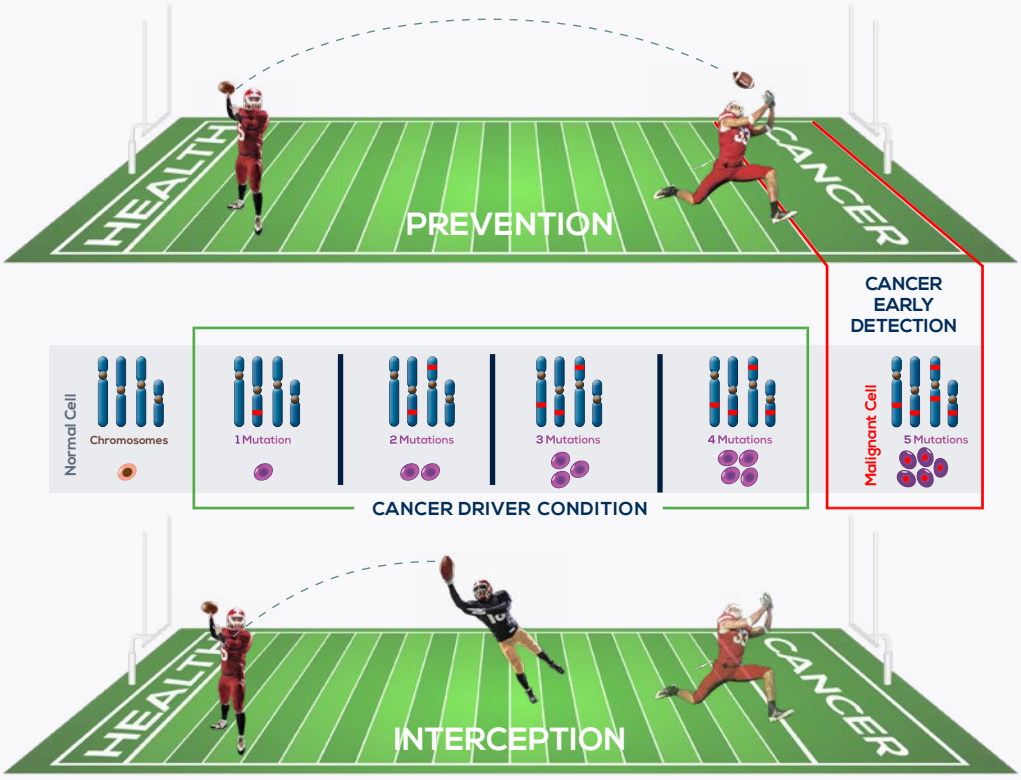
Cancer Driver Interception

Today it is possible to switch the focus from early cancer detection and generic external risk factors reduction to actionable cancer driver interception.

Cancer interception refers to the idea of interrupting carcinogenesis at any point before the development of an invasive disease. Nowadays, most people think that cancer prevention is a passive process that requires avoiding risk factors (e.g. tobacco smoke) to prevent the development of the disease (e.g. lung cancer). This is called “primary prevention”, and



Adapted from: Blackburn EH. Cancer Prev Res (Phila). 2011 Jun;4(6):787-92.

is fundamental. However, in recent years the importance of other approaches has been emerging. In fact, just like cardiovascular disease (CVD) can be intercepted with drugs that reduce CVD risk (such as antihypertensive or cholesterol-reducing drugs), cancer development can be intercepted with risk-reducing agents too. The use of preventive agents that are active against lung cancer that develops in former smokers is a case in point of how cancer can be prevented by new, active approaches, before the advanced disease presents in the clinic.

The idea of CVD interception has been widely accepted. The use of antihypertensive agents in high-risk patients with severe hypertension or with class III/IV heart failure, the use of statins in patients with prior myocardial infarction and very high low-density lipoprotein (LDL) cholesterol, and the use of aspirin in patients with prior MI or stroke are the first success stories in the field. Once effective therapies in advanced disease, now all these strategies are CVD prevention standards.

Instead, up to now the idea of cancer interception has been a hard sell, even among educated people prone to trying active prevention for their personal health. Troubles with adherence to risk reduction-based approaches (e.g. the use of effective breast cancer risk-reducing agents) are around the corner.

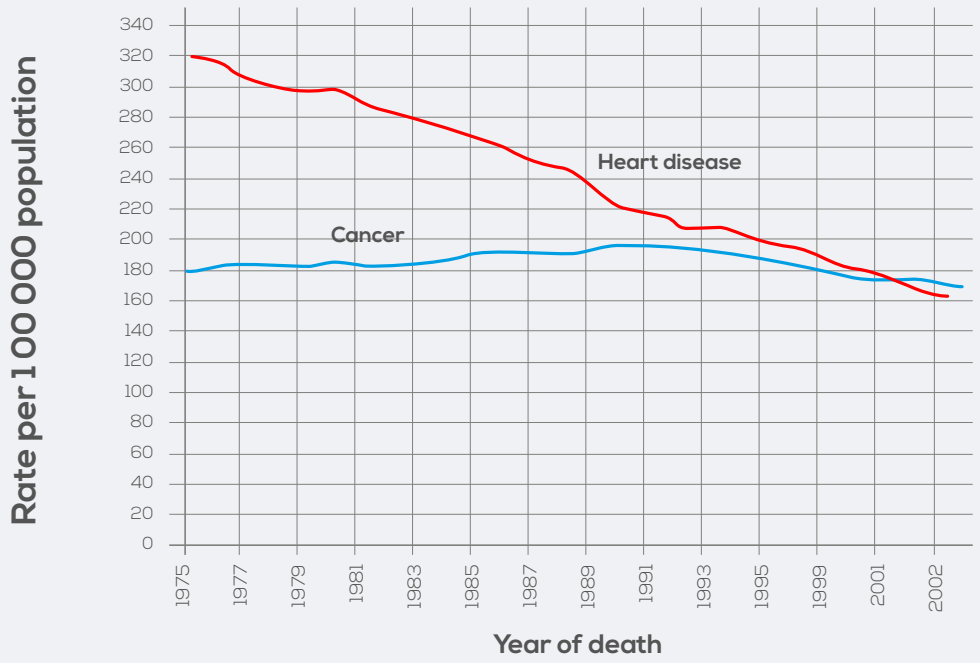
One proposed hindrance is the risk of toxic effects, such as adverse cardiovascular effects produced by nonsteroidal anti-inflammatory drugs (NSAIDs, for example celecoxib) when utilized to intercept colorectal neoplasia. However, low-baseline CVD risk or C-reactive protein level (a well-established marker of inflammation) eliminate this risk, highlighting, at the same time, the importance of a more personalized cancer interception. Interestingly, the use of antihypertensive drugs to reduce CVD is associated with toxicity risk too. The general acceptance of this risk highlights the need to fill the gap between the education on CVD risk reduction and the education on cancer risk reduction.

Another proposed reason behind the resistance to the idea of cancer interception is that whereas CVD risk reduction treats well-known measurable conditions (such as hypertension and high cholesterol level) that can be followed to assess treatment effectiveness, actionable cancer drivers and prodromal conditions are less known by the general population. Actually, there is at least one good example of an actionable measurable condition for cancer too: colorectal adenomas preceding colon cancer. Their number can be reduced by aspirin, which has been shown to reduce the incidence and the mortality from colorectal cancer. Also, surgical control of colorectal adenomas can reduce cancer risk and mortality.

Understandably, people struggle with treating or even curing cancer. However, cancer will never be controlled without prevention. This is why cancer interception is absolutely desirable and necessary.

The disease is a leading cause of death. In 2020, it was responsible for nearly 10 million deaths worldwide. Lung, colorectal, liver, stomach, and breast cancer accounted for half of these losses, and, unfortunately, new diagnosis were common. The new cases were 2.26 million for breast cancer, 2.21 million for lung cancer, 1.93 million for colorectal cancer, 1.41 million for prostate cancer, 1.20 million for skin cancer (non-melanoma), and 1.09 million

for stomach cancer. In the last 40 years, while preventive risk reduction was contributing to the steady fall of heart disease death rates, cancer became the leading cause of death in many US states. In 1999, age-standardized heart disease mortality still was higher than that for cancer in all states, but in 2016 the situation was the opposite in 19 states. Age is among the most important cancer risk factors, and the world population is aging, reaching cancer-prone ages. Moreover, treatment improvement increased cancer patients' lifespan, but survivors' cancer risk is higher. This increasing cancer burden can only be tempered by an approach in which efforts in disease treatment are paralleled by an increase in its interception. This second way is more cost-effective, counts least human costs, and will further reduce cancer burden on public health and wellbeing.



Death rate for cancer and heart disease for ages younger than 85

age-adjusted to the 2000 U.S. standard population. Adapted from: Meyskens FL Jr et al. Cancer Prev Res (Phila). 2011 Mar;4(3):311-23

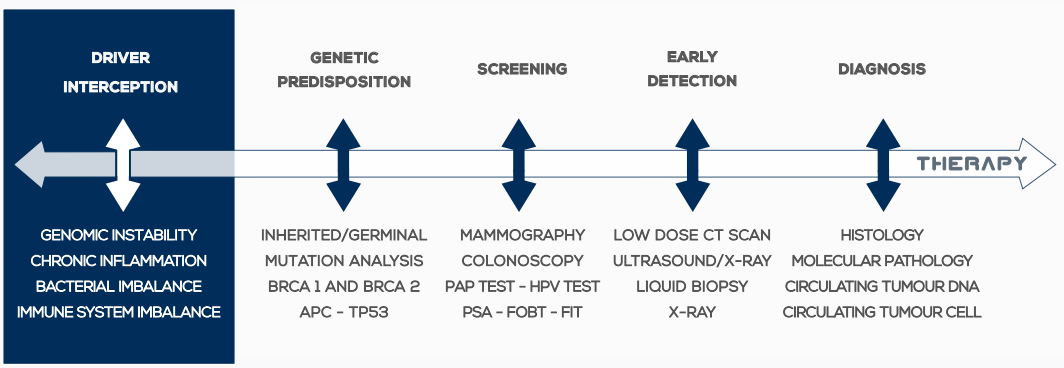
To date, cancer prevention consists of interventions aimed at reducing generic external risk factors (such as smoke, alcohol, unhealthy diet, and radiation), and of early detection. This approach is often perceived as a passive method requiring deprivation (of smoke, of alcohol, of some foods, and so on), and early detection can prevent cancer death but not cancer onset. What is more, people are more and more seeking encouraging health information that could address cancer worry. That means that preventing cancer death (that is, the

target of early detection) is not always the principal focus of people, who sometimes simply feel spontaneously compelled to take action rather than inaction against this disease.

Today it is possible to switch the focus from early cancer detection and generic external risk factors reduction to actionable cancer driver interception. In fact, cancer arises from a process of transformation of normal cells into cancer cells lasting years or decades; during this stage (the so-called prodromal phase) people are apparently healthy and totally asymptomatic, but several factors are actively driving this transformation process. And now we know that just like we can monitor hypertension, hypertriglyceridemia, obesity, and other risk factors that drive CVD development, cancer drivers are measurable too.

Once intercepted, actionable cancer drivers can be monitored, giving people a feedback not only on the progression of the cancer prodromal phase, but also on the effectiveness of the strategies (such as cancer chemoprevention, that is the use of drugs, vitamins or other agents to reduce the risk of cancer development) put in place to counteract their presence – just as in the case of people undergoing regular cholesterol test to evaluate cholesterol-reducing therapy efficiency.

The leading physiological condition that drives cancer development is genomic instability. We all know that genes are strongly involved in determining cancer risk. They are responsible for the hereditary susceptibility to the development of the disease, but not only. In fact, several cancer risk factors are associated with the buildup up of mutations promoting the



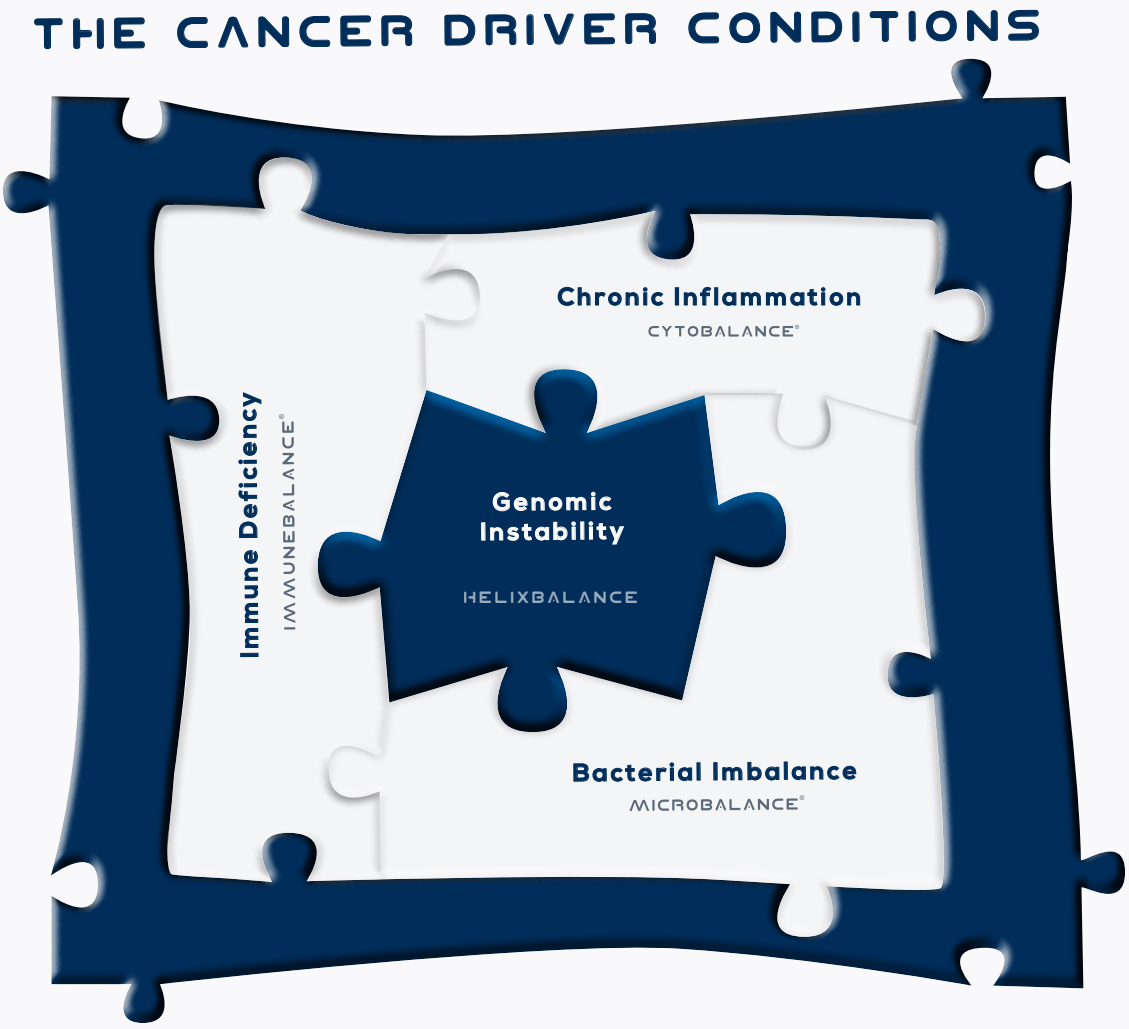
transformation of normal cells into cancer cells. Cells that accumulate such mutations are genetically unstable, and genomic instability is a feature of the cancer prodromal phase.

Other important cancer drivers (chronic inflammation, immune system imbalance, an altered microbiota), provide an environment favorable to the transition from premalignancy to malignancy. They can both promote genomic instability or insist on it, resulting in the amplification of the risk of cancer.

Telomere attrition greatly contributes to genomic rearrangements that can promote cancer

development. Inflammatory cells may produce molecules, such as reactive oxygen species (ROS), that can lead to DNA damage and, as a consequence, to mutations. And immune cell imbalance can halt the ability of the body's natural defenses to kill cancer cells. The human microbiota (that is, the microbial population living on the organism's internal and external surfaces) can play a role too; sometimes, carcinogenesis is linked to the presence of a single bacterial species, whereas in other cases microbiota imbalance (the so-called dysbiosis) are involved.

Bioscience Institute proposes a program that enables actionable cancer driver interception



and management starting from the monitoring of the factors promoting genomic instability, the primary driver of cancer development. The first, crucial, step is the analysis of the sequence of genes involved in the maintenance of genomic stability, such as the ones encoding factors involved in DNA damage response. If mutated, such genes work as cancer

driver factors and their presence should prompt the monitoring of genome instability.

The second step included in the program allow for the simultaneous monitoring of the secondary drivers of cancer: low-grade chronic inflammation, immune system imbalance, and dysbiosis.

For each driver condition, Bioscience Institute developed chemopreventive treatments through which the program completely translate the model of CVD active prevention on cancer driver interception, offering the possibility to act before the onset of cancer to counteract the disease well before early diagnosis, in healthy individuals, and to monitor the efficiency of the lifestyle and chemoprevention-based strategies put in place to halt its development.

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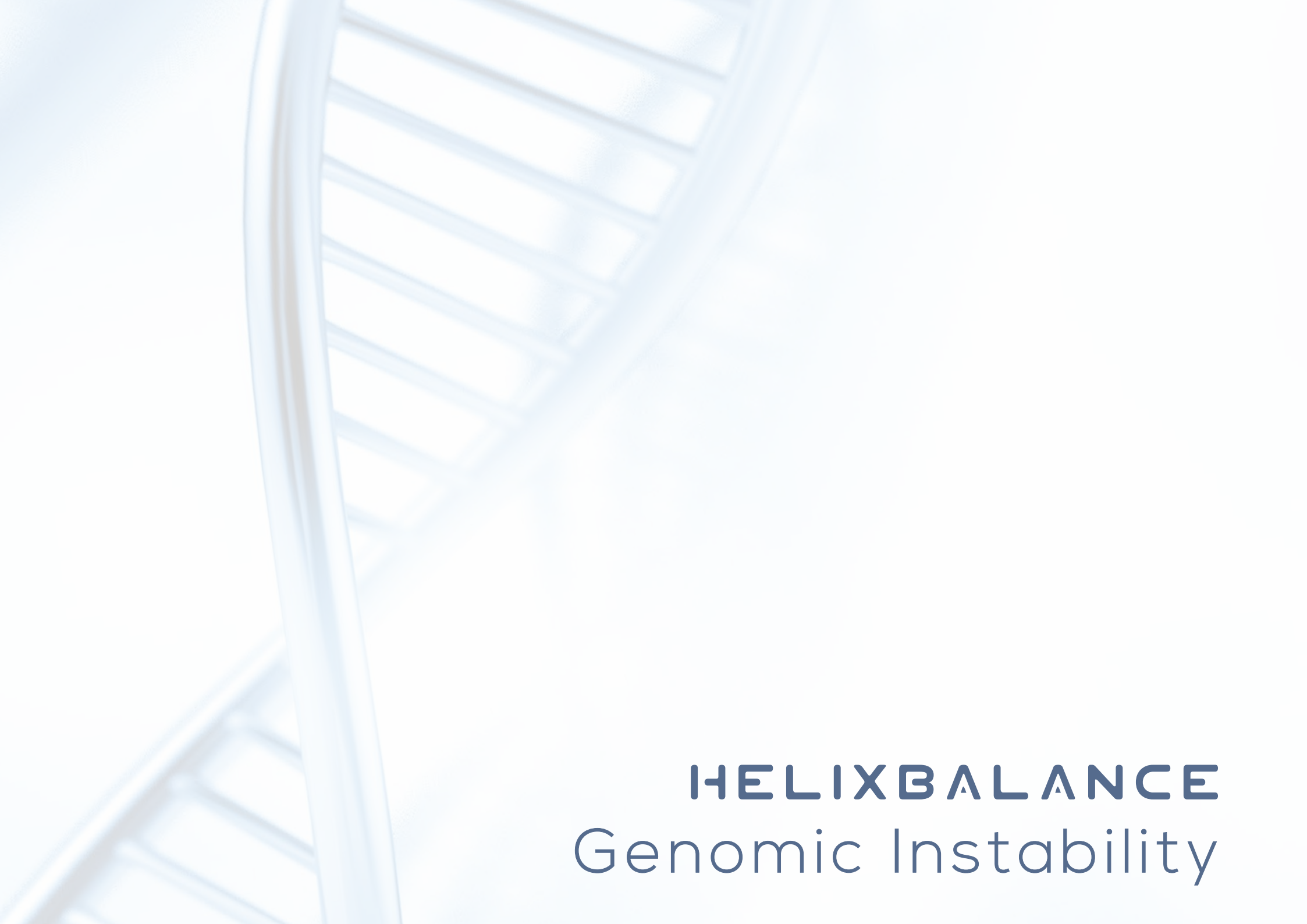
INDEX


HELIXBALANCE
Genomic Instability

HELIXBALANCE

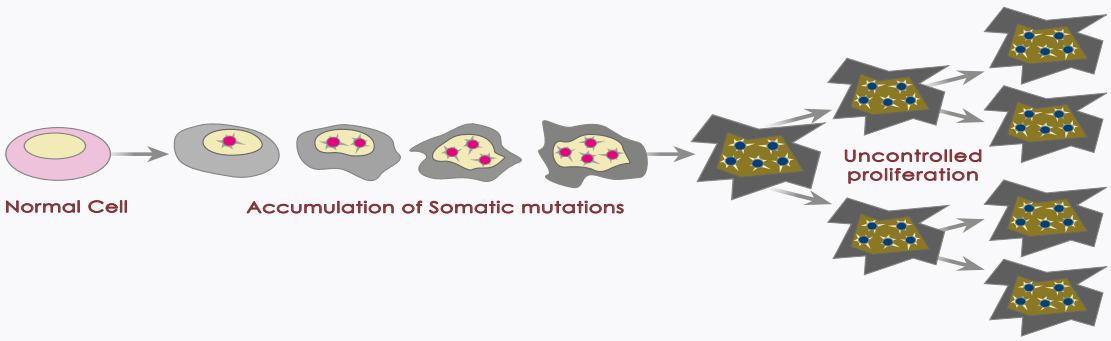
INTRODUCTION

Cancer has its roots in our genes: the genome alterations occurring throughout life, following exposure to other cancer risk factors, make cells grow abnormally, invading tissues and organs, and spreading to other body parts.

Current evidence suggests that between 30% and 50% of all cancer deaths could be prevented and that the biomolecular characterization of cancer is associated with a higher success rate in disease treatment. The interception of genomic instability could help inform decisions on risk factor management and chemoprevention by evaluating the prodromal stage of cancer; that is the stage, lasting several years, during which cells progressively accumulate somatic mutations in clinically healthy individuals showing no cancer symptoms.

HELIXBALANCE, based on the identification of gene mutations that can lead to genomic instability, assess the prodromal, totally asymptomatic, stage of solid cancer.

At the moment, cancer screening tools, even when utilized for prevention purposes, enable solid cancer identification when it is already developed and evident. HELIXBALANCE anticipates early detection several years in advance, allowing interception of the leading cancer driver (genome instability).



GENOMIC INSTABILITY - HOW IT IS LINKED TO CANCER

Genomic instability naturally arises in normal cells through accumulation of genetic and epigenetic (that is, those that do not modify gene sequence) changes. This has been shown to occur over a timespan ranging from years to decades, during which the vast majority of cells that acquired it die, and probably are cleared away by the immune system. However, if an even minimal fraction of cells that have acquired genomic instability are not cleared away, it can eventually give rise to a tumor. Selection pressure toward tumor development can arise from phenomena such as the alteration of the mechanisms that control telomere length or responsible for the DNA damage response (DDR) – as demonstrated by the complex mutational signature of smokers' lung cancer genome or of malignant melanoma, that is cancers that are associated with the exposure to DNA-damaging agents.

Most cancers develop genomic instability at some stage of their progression. In the end, nearly all solid tumors are genetically unstable; or, sometimes, there is a temporary rise in instability, which is followed by the return to a relatively stable genome. Moreover, genomic instability drives intra-tumor genetic heterogeneity, making no tumor identical to another or composed of genetically identical cells.

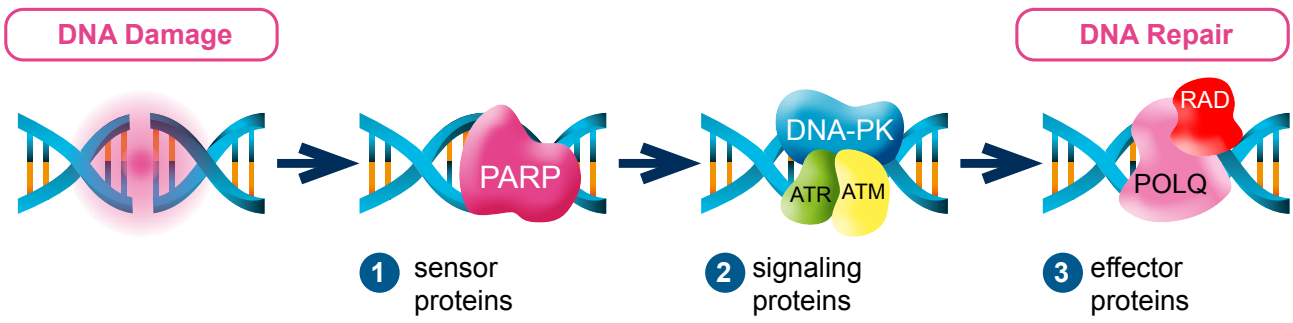
The link between genomic instability and cancer is so strong that upon cancer diagnosis it is possible to take advantage of genomic instability to effectively treat it. For example, evidence suggests that tumors with more single point mutations respond better to immune checkpoint therapies. Also, it is possible to target compromised mechanisms that lead to genomic instability to kill cancer cells without affecting healthy cells. Finally, genomic instability analysis could give an explanation for resistance to therapy and allows for identifying resistance-associated genetic alterations to inform treatment decisions and for monitoring tumor burden in response to therapy.

Several studies have been underlying the mechanisms at the basis of genomic instability. DNA integrity is under constant threat. Damage can arise from both endogenous and exogenous factors, in particular spontaneous DNA damaging events (e.g.: modification of DNA bases, or generation of DNA breaks by reactive oxygen species produced by the normal cell metabolism), exposure to external agents (e.g.: ultraviolet (uv) irradiation, or genotoxic chemicals), and failure in cellular DNA processing and replication. The capability to repair damaged DNA has been shown to be essential to preserving genome stability, and the maintenance of genome integrity by the **DNA Damage Response (DDR)** is critical to preventing cancer development. In fact, germline DNA repair gene mutations are associated with cancer predisposition syndromes (e.g.: BReast CAncer gene – BRCA – mutations with breast and ovarian cancer syndromes), suboptimal repair capacity could influence cancer susceptibility, and several DDR syndromes are associated with increased cancer risk (see Table). Inherited or acquired (germline or somatic) mutations leading to the loss of DNA repair efficiency accelerate the accumulation rate of mutation by 100-1,000 times, and selective pressure favors cancer driver mutations (the so-called "mutator phenotype").

The DDR consist of DNA damage sensors, signal transducers, and protein effectors. The DNA damage sensor machinery can induce apoptosis to eliminate cells that are heavily damaged, or promote DNA damage tolerance. What is more, DDR can activate checkpoints and arrest cell cycle progression to enable DNA repair, promoting genomic stability.

Among the principal signal transducers are ATM (Ataxia-Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3-related protein) kinases. They initiate events that regulate DNA metabolism, cell cycle, and cell fate. Instead, among the enzymatic activities directly involved in DNA repair are nucleases, ligases, polymerases, helicases, topoisomerases, recombinases, glycosylases, kinases, and phosphatases. For example, RAD50, BRCA1 and 2, and Fanconi Anemia (FA) genes are all involved in Homologous Recombination (HR), the DNA repair pathway that can fix double strand breaks (DSBs), the most lethal form of DNA damage.

Mutations in these factors are associated with cancer. For example, ATM is involved in ataxia telangiectasia, a disease which increases lymphoma incidence by roughly 1000-fold. BRCA1 and 2 mutations are linked to breast, ovarian, prostate, pancreatic, and colon cancer. And FA genes are responsible for Fanconi anemia, which is associated with increased breast, ovarian, and oral cancer risk.



DDR SYNDROMES ASSOCIATED WITH CANCER

adapted from Ciccia A and Elledge SJ, 2010

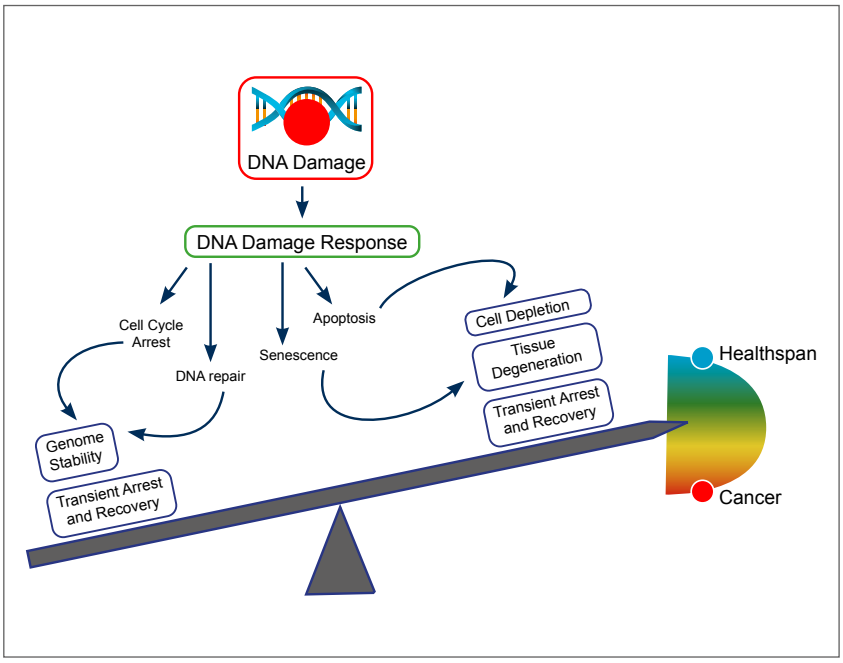
DDR defect(s)	Mutated gene(s)	Syndrome	Cancer(s)
NER	XPA-G, POLH	XPF-ERCC1 (XFE) syndrome	Squamous and basal cell carcinoma, melanoma
HR	BLM	Bloom syndrome	Carcinomas, leukemias, lymphomas
HR, ICL repair	FANCA-C, FANCD1, D2, FANCE-G, FANCI, J, L-N	Fanconi anemia	Acute myeloid leukemia, myelodysplasia, squamous cell carcinoma
HR, damage signaling	ATM, BRCA1, BRCA2, BRIP1, CHK2, NBS1, PALB2, RAD50, RAD51C	Familial breast cancer	Breast cancer, ovarian cancer (BRCA1, BRCA2, RAD51C)
HR, BER, telomere maintenance	WRN	Werner syndrome	Sarcomas
BER, HR?	RECQL4	Rothmund Thomson syndrome	Osteosarcoma, skin cancers
BER, oxidative damage repair	MYH	MYH-associated polyposis	Colorectal cancer
Telomere maintenance	DKC1, TERC	Dyskeratosis congenita	Carcinomas
MMR	MSH2, MSH6, MLH1, PMS2	Hereditary nonpolyposis colorecal cancer	Colorectal cancer, carcinomas
NHEJ	LIG4	Ligase IV syndrome	Acute lymphocyte leukemia, lymphomas
	ARTEMIS	Radiosensitive severe combined immunodeficiency	Lymphomas
Damage signaling, DSB repair, oxidative stress	ATM	Ataxia telangiectasia	Lymphomas, leukemias, breast cancer
Damage signaling, DSB repair	TP53	Li-Fraumeni syndrome	Brain and breast cancer, sarcomas
Damage signaling, DSB repair, replication fork repair	NBS1	Nijmegen breakage syndrome	B cell lymphoma
	ATR, PCTN, SCKL2, SCKL3	Seckel syndrome	Acute myeloid leukemia?

NER, Nucleotide Excision Repair: removal of a small DNA fragment (30 bp) containing lesions such as base dimers and intrastrand crosslinks; **HR**, Homologous Recombination: double strand break repair; **ICL repair**, Interstarnd CrossLink repair: ICLs excision; **BER**, Base Excision Repair: excision of small chemical alterations of DNA bases; **MMR**, MisMatch Repair: replacement of mispaired DNA bases; **NHEJ**, NonHomologous End Joining: double strand break repair; **DSB repair**: Double Strand Break repair.

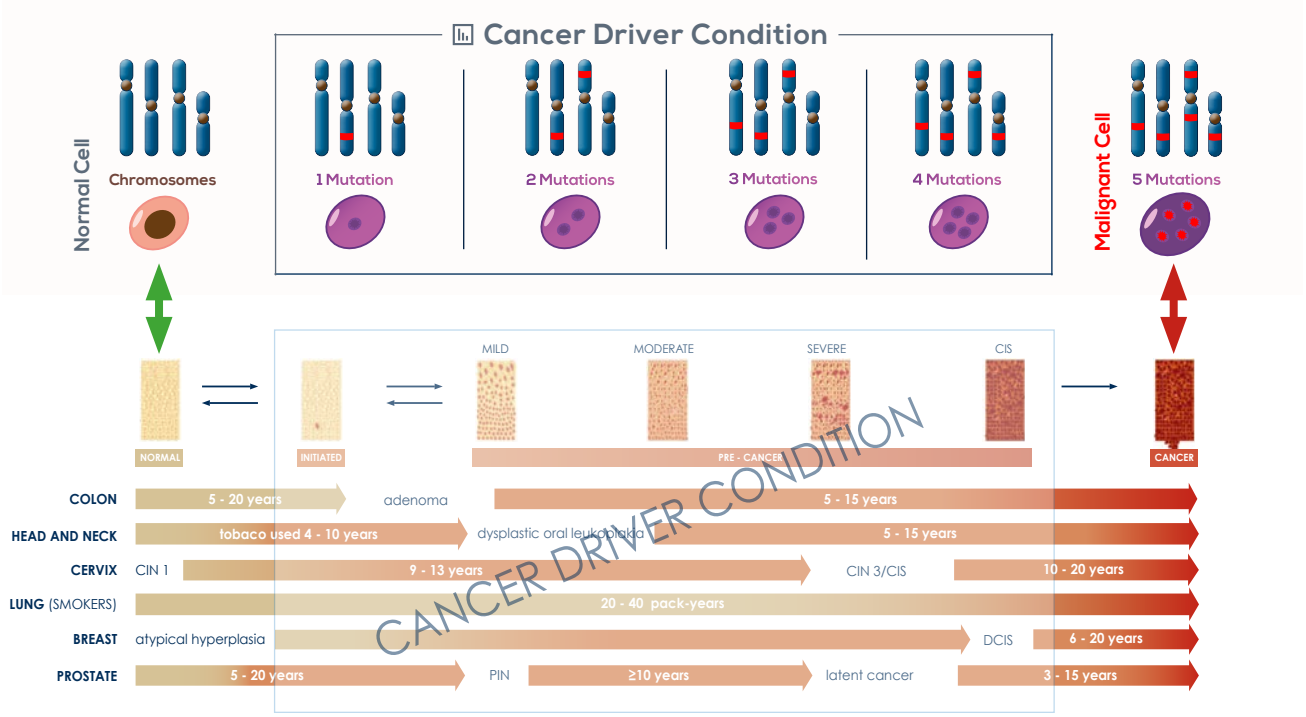
The abnormalities that can arise as a consequence of the failure to repair damaged DNA can be grouped into two distinct forms: **Somatic Point Mutations** (SPMs) and **Somatic Copy Number Alterations** (SCNAs).

SPMs consist of subtle sequence changes. Meanwhile, SCNAs involve gene amplification, loss or gain of whole chromosomes, or chromosome translocations which can result in gain or loss of chromosomal material or in the generation of new gene products. For example, oncogene amplification occurs in a subset of many late-stage cancers, clearly associates with tumor progression, has prognostic significance, provides targets for therapeutics, and represents a common way for cultured cells to acquire resistance to chemotherapy.

Both SPMs and SCNAs are linked to cancer. In general, evidence supports a greater role for SCNAs in developing and maintaining cancer cell population diversity, while uncommon SPMs cause dramatic phenotypes. However, the simple presence of genetic alterations, even when frequent, is not a marker of genetic instability. In fact, by definition, **instability is a matter of rate**. That is why the simple analysis of SPMs or SCNAs cannot be used to determine genetic instability.



Unfortunately, most studies on cancer genomic instability analyze somatic mutations at a single time point, but to accurately estimate somatic mutation rates in an individual, samples from at least two time points are needed. In fact, a single high mutation frequency can be a random error causing incorrect evaluations. Instead, a somatic mutation frequency growth trend, manifested over a prolonged period, is indicative of genomic instability.



SOMATIC MUTATIONS AND CANCER PRODROMAL STAGE

When discussing the relationship between DNA mutations and cancer, it is mandatory to differentiate between **germline mutations and somatic mutations**.

The former are hereditary mutations that have been associated with the risk of cancer development; they occur in germ cells (cells that will give rise to sperm or eggs) and are passed onto every cell of the offspring. BRCA 1 and 2 mutations stand as examples of germline mutations associated with breast cancer susceptibility. In contrast, somatic mutations are acquired, non-heritable, changes in DNA sequence in cells other than germ cells. They occur after conception, cannot be passed onto offspring, and develop in specific tissues (e.g. breast or lung).

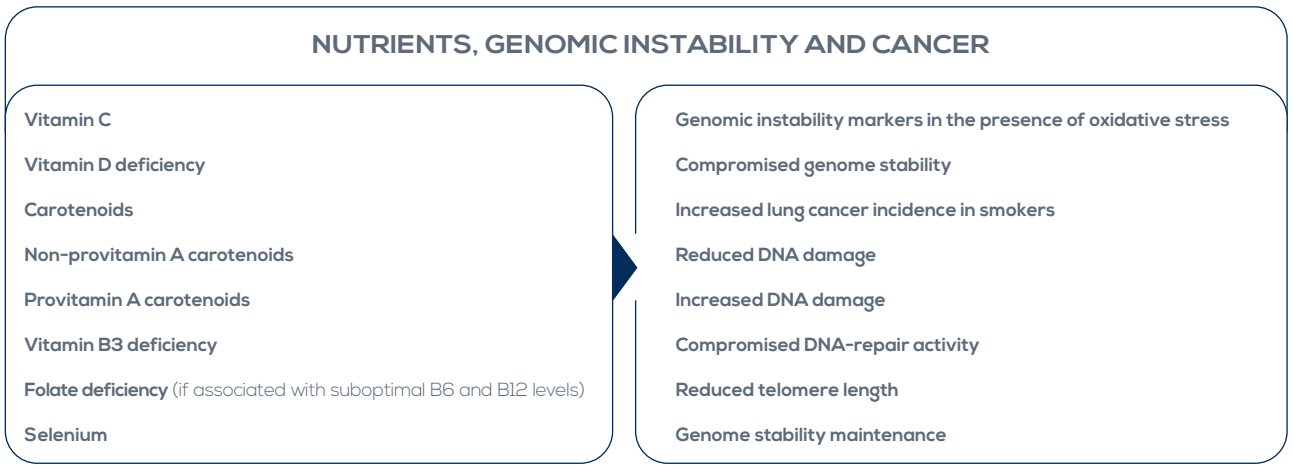
Only a limited percentage of cancers have a clear hereditary component, and even in those cases in which cancer susceptibility is clearly inherited, acquired mutations are needed for cancer to develop. In particular, the progressive accumulation of somatic mutations can lead to cancer and is indicative of genomic instability. Contrary to germline mutations, genomic instability is not a marker of the risk of cancer; rather, it is indicative of the cancer prodromal stage. Researchers already gave proof of concept that genomic instability analysis is useful to assess the cancer prodromal stage. And, fortunately, genomic instability is preventable and actionable.

Nutrigenetics and **nutrigenomics** are interesting tools to avoid genomic instability through a simple approach based on lifestyle. Genome integrity has been shown to be highly sensitive to nutrient status, with optimal nutrient levels differing among individuals. Biomarkers of genome integrity can be utilized to establish recommended daily intakes for nutrients; in turn, optimizing nutrient intake plays a significant role in stabilizing the genome.

For example, in smokers, carotenoid consumption correlates to lung cancer incidence, and β-carotene supplements are associated with a significant increase in mortality; cell cultures studies suggest that any concentration of non-vitamin A carotenoids tends to decrease DNA damage, whereas high concentrations of provitamin A carotenoids such as β-carotene tend to increase it. Vitamin B3 deficiency impairs the function of critical DNA repair enzymes (in particular, PARP proteins), and folate deficiency (especially if combined with suboptimal vitamin B6 and B12 levels) may lead to DNA breaks and telomere shortening. In the presence of oxidative stress, vitamin C correlates with various markers of genome stability. Meanwhile, vitamin D and selenium concentrations are critical in the maintenance of genome stability too. In particular, both vitamin D and selenium possibly protect against chromosomal and telomere aberrations. Cells supplemented with selenium showed reduced DNA breakage, and vitamin D could counteract oxidative stress.

Also, food components can help to increase DDR activity. For instance, resveratrol, which is a polyphenol present in fruits and other vegetable foods (for example in grapes, berries, and peanuts) may activate Sirt1 (sirtuin 1), a DDR repair-activating enzyme. In mice with reduced Sirt1, resveratrol treatment was associated with reduced cancer development.

In a similar way, certain medications are associated with a reduction in genome instability. In particular, when a patient with Barrett's esophagus starts taking NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) their somatic copy number alterations rate drops by an order of magnitude.



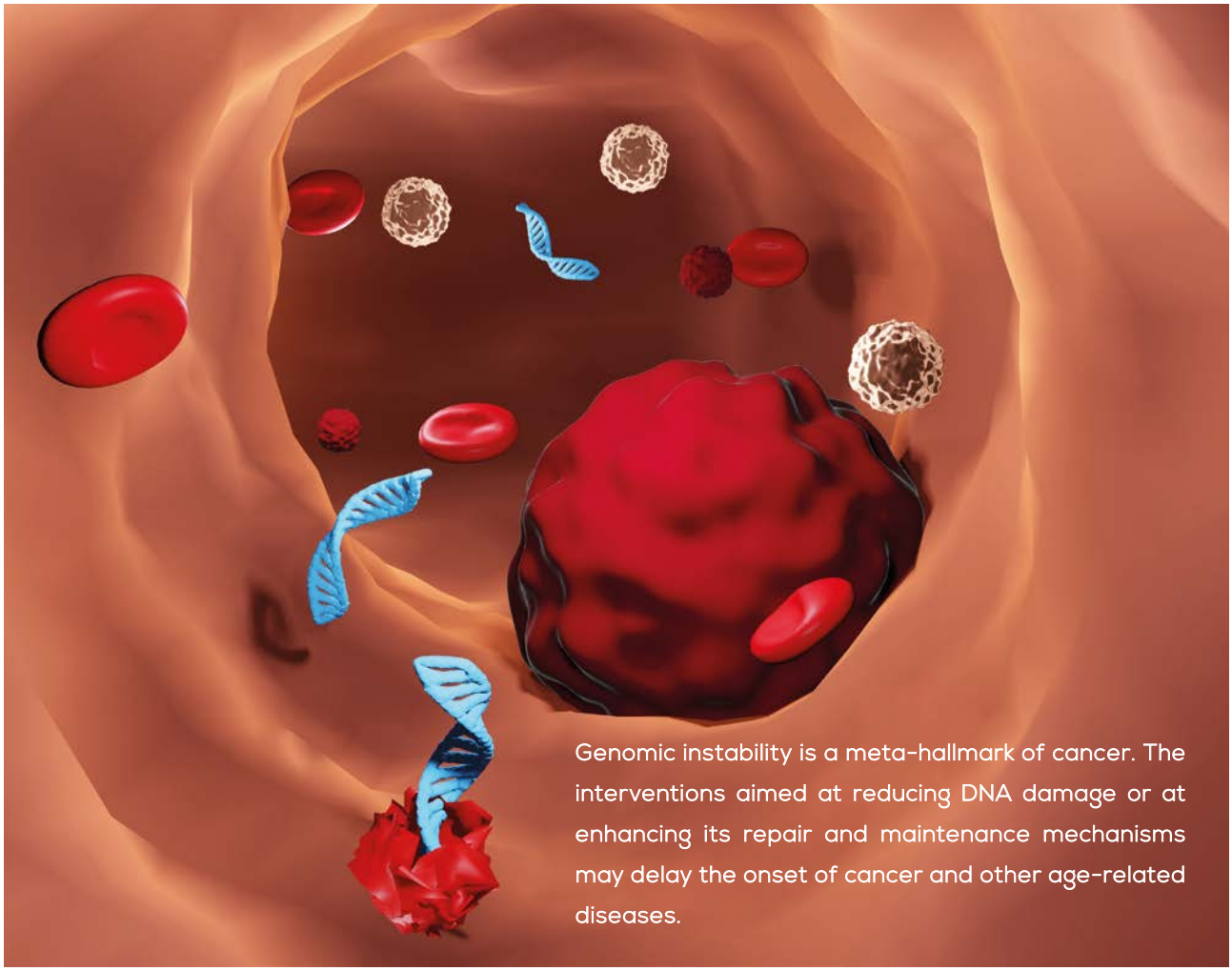
ASSESSING GENOMIC INSTABILITY

Measuring instability is difficult because it requires tracing the evolution of multiple subclones coexisting within a tumor. Hence, monitoring genomic instability by means of traditional tissue-based approaches is, unfortunately, largely unfeasible. Sampling blood can overcome this limit. In fact, compared to healthy people's blood, cancer patients' blood typically present higher levels of total **circulating cell-free DNA (cfDNA).**

Discovered for the first time in 1948, cfDNA consists of small DNA fragments circulating in bodily fluids; it also contains circulating-tumor DNA (ctDNA), but in a limited amount (no more than 10% of the total cfDNA). cfDNA analysis enables detecting early genomic aberrations and cancer cell evolution; however, the low amount of circulating cfDNA and the very low proportion of mutated cfDNA molecules make such an approach challenging.

Taking advantage of the enhanced sensitivity of sequencing methods allowed by the introduction of molecular barcodes (unique sequences utilized to identify unique DNA fragments for a correct interpretation of DNA sequencing) and of other technological advances such as enhanced bioinformatics filtering pipelines, researchers managed to identify clinically relevant genetic alterations in early-stage cancer patients, reaching a sensitivity of less than 1 mutant template molecule per milliliter of plasma. Nevertheless, early detection remains an ambitious clinical application of cfDNA analysis. To establish the sensitivity and specificity required for such an approach, a substantial number of cancer patients and healthy controls is needed; furthermore, sources of false positive results exist. In contrast, monitoring overall genomic instability in healthy individuals, rather than the occurrence of a single driving genomic event, could help identify people that might enter into early access screening prevention programs.

In a proof of principle study, researchers at the University Hospital Basel, the University of Trieste, the Memorial Sloan Kettering Cancer Center in New York, Tor Vergata University in Rome, and Bioscience Institute in San Marino demonstrated the technical feasibility of extracting and analyzing healthy individuals' cfDNA to study genomic alterations.

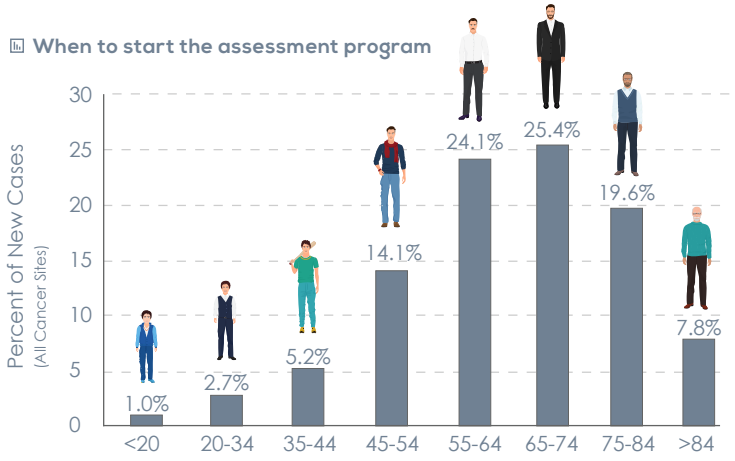


Genomic instability is a meta-hallmark of cancer. The interventions aimed at reducing DNA damage or at enhancing its repair and maintenance mechanisms may delay the onset of cancer and other age-related diseases.

ANALYZING cfDNA TO ASSESS GENOMIC INSTABILITY

Alborelli *et al.* studied genomic alterations by means of molecular barcoded ultra-deep sequencing. They first analyzed tissue and blood samples from patients with a histologically confirmed diagnosis of cancer (30 cases of non-small cell lung cancer and 8 cases of breast cancer). Their results highlighted a substantial level of concordance (71%) between blood and tissue mutation profiles, suggesting that **cfDNA analysis reliably mimics tissue genomic features.** Moreover, certain mutations were detected only by blood cfDNA analysis, confirming the potential clinical value of using this approach in parallel to tissue biopsy, particularly for detecting mutations that are relevant for acquired therapy resistance.

Then, they analyzed cfDNA in the blood of 106 women that were healthy at the time of sample collection and that were followed up regularly for the development of breast cancer or any other malignancy for up to 10 years later. A first group of participants did not develop any malignancy during the follow-up; a second group developed fibrocystic breast changes (for example, mastopathy); a third group developed breast cancer, and a fourth group another type of solid tumor. Most healthy individuals (84%) showed no genetic alterations, but 7 out of 55 healthy individuals analyzed showed clinically relevant gene mutations, 4 of which are well known cancer hotspot mutations.



AGE Advancing age is the most important risk factor for cancer development overall, and for many individual cancer types. The median age of a cancer diagnosis is 66 years but the disease can occur at any age. SEER 18 2007-2011.

HELIXBALANCE

In this scenario, Bioscience Institute presents **HELIXBALANCE**, a five-test program allowing for the interception of genomic instability drivers and cancer prodromal stage assessment. Monitoring both somatic and germline mutations, HELIXBALANCE allows for identifying mutations that promote genome instability and obtaining a trend of stability for solid cancer associated mutations.

All that is required is a 10-20 cc blood sample each year, from which cfDNA is obtained. The mutation rate in cfDNA is analyzed by means of Multi Biomarker Next Generation Sequencing (NGS) and a sophisticated management software. NGS is an innovative DNA sequencing technology that allows for sequencing a high number of small DNA fragments at the same time, with a very high coverage of a region of interest – especially important for identifying mutations associated with cancer that are present at low fractions. Based on the HELIXBALANCE algorithm, if there are no mutations in cfDNA, or if mutations in cfDNA are also detected in white blood cells' DNA (germline control), all that is needed is to schedule the next check-up in one year; in fact, the presence of a mutation in the germline control means that it is an inherited (not acquired) mutation, not indicative of genome instability. However, if mutations in cfDNA are not detected in the germline control, that means they are somatic, acquired mutations. In such a case, germline mutations in cancer susceptibility genes should be also tested, looking for genes that can promote cancer development in the presence of somatic mutations.

In the end, patients are referred to a counseling session with a cancer specialist. Genetic instability can be further analyzed by HELIDX, a non-invasive screening assay for early detection of solid cancer. This assay is based on simultaneous NGS analysis of ctDNA, circulating tumor cell DNA (CTCs), and white blood cell DNA (germline control) obtained from healthy and asymptomatic subjects. The first level **HELIXAFE** prevention program is meant to identify mutations responsible for genome instability. This test is called **HELIXBALANCE** and analyzed genes include factors involved in DNA damage and/or repair, chromatin remodeling or DNA methylation, β-catenin/WNT signaling, and cell cycle control. If no mutation is detected, individuals are sent to genetic counseling to plan the next check-up, whereas if one or more mutations are detected in the cfDNA but not in WBC, individuals are sent to genetic counseling to plan a second level prevention program.

There are 4 possible second level cancer prevention programs:

In the past, cancer prevention was achieved by means of the analysis of familial susceptibility, and test results needed to be interpreted, potentially resulting as non-informative; the analysis and monitoring of mutation rates are instead objective parameters. Aging is the most important cancer risk factor, but the disease can occur at all ages, and it is never too soon or too late to start prevention by evaluating genomic instability.

HELIXAFE NGS PANELS

MY CHECK-F

HEREDITARY INSTABILITY

85 GENES — SENSITIVITY 99%

ALK	BRCA2	EPCAM	GREM1	MSH6	NTRK1	PTCH2	SDHB	TSC1
APC	BRIP1	EXT1	KIT	MUTYH	PALB2	PTEN	SDHC	TSC2
ATM	CDC73	EXT2	MAX	MC1R	PDGFRA	RAD50	SDHD	VHL
AXIN2	CDH1	ENG	MEN1	MDH2	PHOX2B	RAD51C	SMAD4	WT1
ACVRL1	CDK12	EPAS1	MET	MITF	PMS1	RAD51D	SMARCA4	XPC
BAP1	CDK4	FANCG	MLH1	MSR1	PMS2	RB1	SPINK1	
BARD1	CDKN1B	FH	MLH3	NBN	POLD1	RET	STK11	
BLM	CDKN2A	FLCN	MRE11A	NF1	POLE	RHBDF2	SUFU	
BMPR1A	CHEK2	FANCC	MSH2	NF2	PRSS1	SDHA	TMEM127	
BRCA1	CFTR	GALNT12	MSH3	NTHL1	PTCH1	SDHAF2	TP53	

MY CHECK-M

HEREDITARY INSTABILITY

90 GENES — SENSITIVITY 99%

ALK	BRCA1	CFTR	FLCN	MLH1	MSR1	PMS2	RB1	SPINK1
APC	BRCA2	EPCAM	FANCC	MLH3	NBN	POLD1	RET	STK11
ATM	BRIP1	EXT1	GALNT12	MRE11A	NF1	POLE	RHBDF2	SUFU
ATR	CDC73	EXT2	GEN1	MSH2	NF2	PRSS1	SDHA	TMEM127
AXIN2	CDH1	ENG	GREM1	MSH3	NTHL1	PTCH1	SDHAF2	TP53
ACVRL1	CDK12	EPAS1	HOXB13	MSH6	NTRK1	PTCH2	SDHB	TSC1
BAP1	CDK4	FAM175A	KIT	MUTYH	PALB2	PTEN	SDHC	TSC2
BARD1	CDKN1B	FANCA	MAX	MC1R	PDGFRA	RAD50	SDHD	VHL
BLM	CDKN2A	FANCG	MEN1	MDH2	PHOX2B	RAD51C	SMAD4	WT1
BMPR1A	CHEK2	FH	MET	MITF	PMS1	RAD51D	SMARCA4	XPC

HELIXBALANCE

Genomic Instability
Genes Driver

28 GENES

LIMIT OF DETECTION 0,2 %

APOBEC3B	ATM	BARD1	CDK12	DICER1	MLH1	PMS2
ARID1A	ATR	BLM	CDKN2A	FANCD2	MSH2	RAD21
ARID1B	ATRX	BRIP1	CHEK2	FOXA1	MSH6	RECQL4
ARID2	AXIN1	BUB1B	CTNNB1	KMT2D	NFE2L2	TP53

HELIXMOKER

Lung Cancer
Genes Driver

11 GENES - 169 HOTSPOTS

SENSITIVITY 100%

ALK	KRAS	PIK3CA
BRAF	MAP2K1	ROS1
EGFR	MET	TP53
ERBB2	NRAS	

EGFR:	T790M, L858R, EXON19 DEL, C797S
KRAS:	G12X, G13X, Q61X
ALK:	1151 TINS, L1152R, C1156Y
BRAF:	V600E

HELIXCOLON

Colon Cancer
Genes Driver

14 GENES - 245 HOTSPOTS

SENSITIVITY 99,9%

AKT1	KRAS
BRAF	MAP2K1
CTNNB1	NRAS
EGFR	PIK3CA
ERBB2	SMAD4
FBXW7	TP53
GNAS	APC

KRAS/NRAS:	G12/G13/Q61
BRAF:	V600E
PIK3CA:	E545K, H1047R
TP53:	R175H R273H/C/L
APC (including p.R876*, p.Q1378*, and p.R1450*)	
SMAD:	R361C/H
CTNNB1:	S45F, T41A

HELIXGYN

Breast Cancer
Genes Driver

10 GENES - 157 HOTSPOTS

SENSITIVITY 99,9%

AKT1	FBXW7
EGFR	KRAS
ERBB2	PIK3CA
ERBB3	SF3B1
ESR1	TP53

PIK3CA:	E545K AND H1047R
AKT1:	E17K
ESR1:	ANTI-ESTROGEN RESISTANCE
TP53:	LOSS OF FUNCTION
ERBB2:	SENSITIVITY TO ANTI-ERBB2 THERA

HELIXPAN

Pan-Cancer
Genes Driver

52 GENES

SENSITIVITY 99,9%

AKT1	CTNNB1	ERG	FLT3	S45F	MAP2K1	MTOR
CCND3	BRAF	ESR1	GNA11	T41A	PDGFRA	RAF1
ALK CDK4	DDR2	ETV1	GNAQ	KIT	SMO	MYC
APC;	CCND1	FBXW7	GNAS	NTRK1	MAP2K2	RET
CDK6;	EGFR	FGFR1	HRAS	SF3B1	PIK3CA	NRAS
AR	CCND2	FGFR2	IDH1	KRAS	TP53	ROS1
CHEK2	ERBB2	FGFR3	IDH2	NTRK3	MET	
ARAF	ERBB3	FGFR4	R361C/H	SMAD4	PTEN	

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CYTOBALANCE
Chronic Inflammation

CYTOBALANCE

INTRODUCTION

For many years, inflammation has been referred to as the response of the organism to tissue injury and infection. However, lots of things have changed since Aulus Cornelius Celsus (ca 25 BC – ca 50 AD) first defined acute inflammation as a state characterized by the presence of swelling, pain, redness and warmth - hence the name, derived from “flame”.

In the 19th century the father of modern pathology, Rudolph Virchow, concluded that there were various inflammatory processes. Today, it is known that inflammation can be induced by tissue stress and malfunctioning while in the absence of infections or overt tissue damage. In particular, low-grade chronic inflammation is triggered by sentinel immune cells that monitor for tissue stress and malfunctioning. It has also been associated with a number of lifestyle factors (such as smoke, unhealthy diets, sleep deprivation, and low levels of physical activity) and new correlations are constantly emerging.

Among the health issues associated with low-grade chronic inflammation is cancer. Years of research led to the conclusion that immunity serves both as a tumor suppressor and as an initiator or promoter of the disease, and that chronic inflammation is among the factors responsible for these associations. Moreover, chronic inflammation causes genomic instability. Therefore, evidence of genomic instability provokes the need to analyze the presence of inflammation as both a possible cause of the observed instability and a cancer-promoting factor.

It is currently recognized that inflammation is present when the concentration or the activity of elements involved in innate responses, such as pro-inflammatory cytokines, is increased. CYTOBALANCE is the inflammation monitoring program that makes finding out and correcting the increase of these molecules possible.

Through the periodic monitoring of blood cytokine levels in apparently healthy people, CYTOBALANCE allows for intercepting an important cancer driver well before disease development and taking action against it.

THE CAUSES OF INFLAMMATION

Different individuals develop low-grade chronic inflammation in different ways. The inflammatory tone can increase progressively during several years or decades, depending on genetics, anatomical features, immunological history (early critical immunological experiences – such as intrauterine stimuli, type of birth, neonatal feeding, and the early use of antibiotics – and events in adulthood – such as infections), and lifelong lifestyle habits (e.g. diet).
Macromolecular damage, metabolism, epigenetics, stress, proteostasis, and stem cell regeneration are all interconnected factors influencing this peculiar state of inflammation. Their effect becomes more and more evident with aging, when the chronic activation of the innate immune system can become damaging, giving rise to a form of inflammation occurring in the absence of infection that is called "**inflammaging**".

Inflammaging is primarily driven by endogenous signals, including the presence of cell debris, misplaced self-molecules, and misfolded or oxidized proteins. There are several cellular and molecular mechanisms involved: cellular senescence; the dysfunction of mitochondria (the powerhouses of the cell); defective mechanisms of autophagy and mitochondria degradation; the activation of the intracellular multiprotein complex that detects biological and non-biological stressors (the inflammasome); the dysregulation of the system controlling protein degradation; the activation of the DNA damage response; changes in the composition of the microbiota (dysbiosis); and nutrient excess and overnutrition, which can generate a specific type of chronic inflammation called "**metaflammation**" associated with metabolic diseases such as obesity and type 2 diabetes.
All these stimuli converge on a small number of sensors which trigger the innate immune response causing inflammation and an adaptive metabolic response. This process is critical for survival until middle age, but with aging the inflammatory response usually increases, becoming detrimental – and eventually leading to chronic inflammation – in the post-reproductive age. The accumulation of senescent cells, the hyperactivation of immune response, and, according to the garbage accumulation theory, the age-related progressive impairment of cell debris elimination systems largely sustains this phenomenon.
Metaflammation contributes to the onset of insulin resistance, activating inflammatory responses that affect organs such as liver and adipose tissue. This situation leads to an increased risk for metabolic syndrome, which, in turn, is associated with increased cancer risk. Lipids play a central role in this process, increasing oxidative stress and cytokines such as IL-6. After a high-fat meal, blood levels of lipopolysaccharide (LPS, the endotoxin associated with sepsis) increase too; this generates a state called **metabolic endotoxemia** associated with low-grade inflammation. Finally, high-fat diets can alter gut microbiota, further increasing LPS production.
Dysregulation in meal timing contributes to these metabolic and inflammatory alterations, whereas nutrient-dense diets induce the increase of adipocyte size, until they reach a structurally critical condition that contributes to metaflammation.

Another inflammation-causing agent is **mitochondrial DNA** (mtDNA) that is released from mitochondria.
Mitochondria evolutionarily derive from energy-producing bacteria which have been engulfed by ancestral cells approximately 2 billion years ago. That is why its constituents (including freely circulating mtDNA) are immunogenic in a way similar to bacterial molecules – and that is why when released into the cytosol and extracellular environment they trigger innate immune responses, promoting inflammation. In particular, the pro-inflammatory action of mtDNA depends on its oxidation. The highly oxidative extracellular environment typical of chronic inflammatory diseases may overwhelm antioxidant systems, further enhancing its pro-inflammatory potential.
Plasma mtDNA levels are strongly variable between individuals. Almost 20% of this variability is explained by a familial component. Other factors potentially influencing it are mutations or drugs affecting intracellular mtDNA copy number, immune mechanisms controlling the response to inflammatory triggers, and the propensity to cell death, which can be enhanced in response to common chronic viral infections, such as cytomegalovirus infections in southern European populations. Moreover, mtDNA plasma levels gradually increase after the fifth decade of life. This association is not a mere epiphenomenon: the increase in plasma mtDNA can contribute to the onset and maintenance of the pro-inflammatory status typical of inflammaging.
Higher mtDNA plasma levels correlate with higher amounts of pro-inflammatory cytokines, in particular Tumor Necrosis Factor TNF-α and IL-6. Also, blood mtDNA is significantly higher in a number of cancers, including ovarian, testicular, prostate, bladder, renal cell and lung cancer.

POSSIBLE CAUSES OF CHRONIC INFLAMMATION

Infections, psychosocial stressors, aging

Health issues (e.g. pre-hypertension, obesity, T cell dysfunction, leaky gut)

Lifestyle (smoke, diet, sleep deprivation, lack of physical activity)

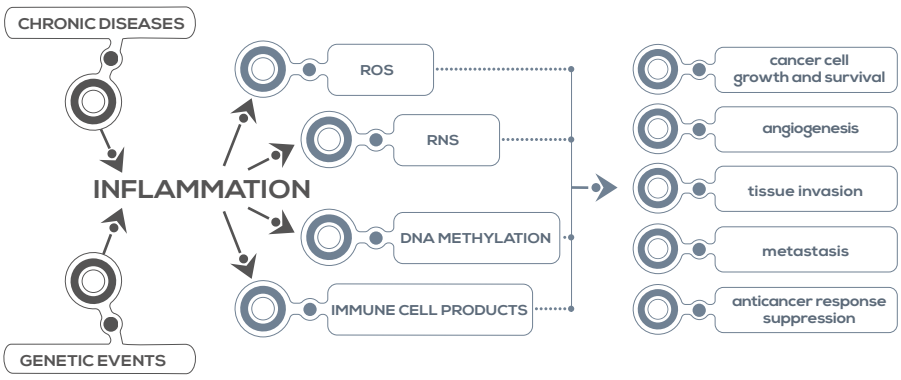
THE ROLE OF INFLAMMATION IN CANCER

More than 20% of all cancers are initiated or exacerbated by inflammation. For example, hepatocellular carcinoma, gastric cancer, and colon cancer have all been associated with chronic inflammation. Gastritis, the inflammation of the lining of the stomach, correlates with gastric cancer, whereas colitis, the inflammation of the colon, associates with colon cancer.

Also, non-infectious causes of chronic inflammation, such as hormonal changes, cigarette smoke and other toxicants (for example adhesives, air fresheners, cleaning products, and glue) increase cancer risk. It is estimated that almost 20% of smokers with inflammation of their bronchial tubes' mucous membranes (a bronchitis) can develop cancer. Moreover, specific variants of inflammatory genes such as tumor necrosis factor (TNF) and IL-1 and mutations in genes playing important roles in innate immunity have been associated with cancer. For example, the aberrant activation of NF-kB or STAT3, which are involved in inflammation control, is found in over 50% of all cancer; this activation renders both premalignant and fully transformed cells resistant to apoptosis, and accelerates their rate of proliferation, increasing tumor growth.

On the other hand, non-steroidal anti-inflammatory drug (NSAIDs) use has been associated with a 30-60% reduction of colon cancer incidence.

In general, pro-inflammatory cytokines have been implicated in inflammation-associated tumors. This association is supported by epidemiological, genetic, and pharmacological studies. Inflammation can contribute to cancer initiation through mutations in genes involved in cell division, survival, senescence, or genomic instability (e.g. genes involved in error correction after DNA replication), or promoting chromosome instability and DNA double strand breaks. It can also promote cancer development through cellular and extracellular signals making cells resistant to growth-inhibitory signals, apoptosis, or antitumor immunity. It acts both via extrinsic pathways (that is, via chronic conditions associated with smoldering, non-resolving inflammation) and intrinsic pathways (that is, genetic events that orchestrate the generation of cancer-related inflammation), playing a crucial role in several aspects of tumor development, including cellular transformation, cell survival, cell proliferation, invasion, metastasis formation, and angiogenesis.



A key event in inflammation-related carcinogenesis is the production of **reactive oxygen and nitrogen species** (ROS and RNS). These highly reactive molecules **damage DNA** promoting cancer development (e.g. cholangiocarcinoma related to liver fluke infection), can induce irreversible and irreparable protein changes, and can contribute to the generation of reactive lipids that can further interact with and damage DNA and proteins. All these lesions result in molecule disfunction, damaging tissues and activating stem cells for tissue regeneration. Moreover, ROS and RNS damage stem cells too, resulting in mutation accumulation and, eventually, cancer stem cell generation. Damage to cancer stem cells' DNA is associated with more aggressive cancer.

Another pivotal event is **DNA methylation** – the addition of a chemical group (methyl) to specific DNA sequences. ROS and RNS induce global DNA hypomethylation, resulting in **genomic instability**, and inflammatory cytokines such as IL-6 affect the expression of the gene encoding for DNA methyltransferase 1 (DNMT1), enhancing, for example, the methylation of tumor suppressor genes – thus downregulating their expression, an event that can represent the first step of carcinogenesis.

Finally, chronic inflammation is associated with **immune suppression**. Ongoing inflammatory responses can suppress anticancer immune responses. In fact, tumor-associated immune cells can produce immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF-β) and other molecules that negatively influence immune responses against cancer cells.

CANCER-ASSOCIATED CAUSES OF INFLAMMATION

- Genetics, age, tobacco smoke, toxic molecules, changes in hormone levels
- Mutations or drugs affecting mtDNA copy number
- Immune mechanisms controlling proinflammatory factor response
- Increased programmed cell death associated with chronic viral infections (e.g. Cytomegalovirus infection)

THE ACTIONABLE BIOMARKERS OF INFLAMMATION

The ultimate goal of every type of inflammation is to return tissues to their normal state, and acute and chronic inflammation share many of the same effector molecules and cells involved in this task.

Among other factors, **pro-inflammatory cytokines** are produced by activated inflammatory cells. If limited and short-lived, their expression is beneficial; however, the long-term expression of cytokines during chronic inflammation is detrimental and leads to chronic diseases, including cancer.

Another important tumor-promoting factor is **Tumor Necrosis Factor (TNF)**. It is produced during the initiation of inflammatory responses, plays a critical role in chronic inflammation maintenance, and is involved in tumor growth, angiogenesis, tissue remodeling, and metastasis. In breast carcinoma, for example, TNF-α serum concentration has been associated with a more advanced tumor phenotype.

Elevated expression of **IL-17** and of another cytokine playing an important role in its expression, **IL-23**, has been detected in colon, ovaries, lung, breast, stomach, skin, liver, and head and neck cancer. Moreover, in small cell lung cancer IL-17 expression has been associated with tumor stage and metastases number, and it could be a new prognostic biomarker.

These inflammatory cytokines along with others, can be upregulated and lead to exacerbation of tumor progression. They are increased in chronic pancreatitis (a condition associated with pancreatic cancer) and in early stages of cancer. IL-8 levels in chronic pancreatitis are 9.3 times more elevated than in normal pancreatic tissue, and in pancreatic cancer some polymorphisms in the IL-6 gene make its levels increase. IL-1β is involved in tumor growth and metastasis, and TNF-α can be used as a target for pancreatic cancer treatment. Intriguingly, IL-10 plays a dual role: it prevents growth of the tumor, but it is upregulated in patients with pancreatic cancer and is correlated with tumor stage and prognosis.

Elevated expression of IL-6, IL-17 and IL-23 has been linked to adverse colorectal cancer prognosis and to a more aggressive disease, and IL-6 participates in the growth of multiple myeloma cells too. Its elevated expression is also linked to increased risk of colorectal cancer, and in general its expression in cancer correlates with a poor prognosis. Together with TNF, IL-6 promotes tumor development through direct effects on premalignant cells and by creating a tumor-promoting environment.

Furthermore, the increased concentration of the acute phase protein **C-reactive protein** (CRP) is regarded as a synonym for inflammation, even when only modest. CRP is produced by liver cells in response to circulating pro-inflammatory cytokines such as IL-6. Low-grade inflammation is associated with minimally elevated CRP levels (3-10 mg/l) compared with those associated with tissue injury or emerging infection. Such modest CRP increases are found in a consistent proportion of the western population (e.g. in 30% of Americans) and are stable markers of inflammation usually unaffected by physiological or pathological processes other than underlying inflammation.

Serum CRP correlates with progressive disease and decreased survival in esophageal, gastric, colorectal, liver, pancreatic, ovarian, and other cancers. Moreover, elevated CRP is associated with a higher risk of developing any cancer. For example, prediagnostic levels of CRP have been associated with increased colorectal cancer incidence and mortality, and with breast cancer risk.

CANCER	CYTOKINE	LINK
Breast cancer	IL-1β, TNF-α IL-6	Advanced stage Increase associated with disease progression
SCLC	IL-17 IL-10	Stage of the disease and metastasis number Angiogenesis
Stomach cancer	Several proinflammatory cytokine genes IL-10, IL-12, IL-18 IL-1β, IL-18 IL-6 TGFβ	Polymorphisms associated with cancer risk Disease progression Cancer cell proliferation Cancer depth, venous and lymphatic, metastasis, poor outcome Direct suppression of cancer and of its progression
Ovarian carcinoma	Cytokine level IL-1β, TNF-α, IL-6, IL-8 IL-1β, TNF-α, IL-8 TGF-β IL-6, IL-8	Increase associated with survival reduction Increased expression in cancer cells Angiogenesis and metastasis Transcription of genes involved in tissue invasion and in cell migration Chemoresistance
Pancreatic cancer	IL-1β, TNF-α, IL-6, IL-8, TGF-β IL-1β IL-10	Increased levels can promote cancer progression Metastasis Cancer growth prevention
Colorectal cancer	IL-6, IL-17, IL-23 IL-6 IL-6, TNF	More aggressive cancer, poor prognosis Increased risk Cancer development
Multiple myeloma	IL-6	Cancer growth

LIFESTYLE AGAINST INFLAMMATION: ROLE OF NUTRACEUTICALS

Lifestyle factors can modulate the production of inflammatory molecules, but not exclusively. They can also induce the production of ROS, which in turn can induce inflammation by regulating molecules such as pro-inflammatory transcription factors.

Specifically, nutraceuticals play an interesting role in suppressing inflammatory pathways. They are food constituents with potential health benefits other than their nutritional value. They can be isolated from foods and sold in the form of dietary supplements.

Nutraceuticals potentially useful against inflammation include caffeic acid phenethyl ester (CAPE), capsaicin, emodin, epigallocatechin gallate (EGCG), guggulsterone, sanguinarine, deguelin, quercetin, ginseng, ginger, vitamins C and D, gamma linoleic acid (GLA), gentianine, and bromelain. Among others, curcumin and resveratrol seem particularly interesting.

Curcumin is a polyphenol derived from the turmeric spice (*Curcuma longa*), and it is the yellow component of curry. It can hinder cancer cell production and promote apoptosis by decreasing the production of p53, a protein mutated in over 50% of cancers, and of NF-kB. In a murine model of ovarian cancer, it was found to suppress the STAT3 pathway.

Curcumin supplementation leads to a significant decrease in CRP levels, demonstrating an anti-inflammatory effect. Its action is based on multiple mechanisms that modulate the production and the activity of several molecules playing a role in inflammation (transcription factors such as NF-kB, cytokines such as IL-6, IL-12, and TNF-α, and protein kinases). It can also directly bind and inhibit cyclooxygenase COX-1 and COX-2 – the pharmacological targets of nonsteroidal anti-inflammatory drugs (NSAIDs) – and matrix metalloproteinases (MMPs) – a family of enzymes expressed in pathological conditions involving inflammation. Finally, curcumin is an antioxidant that suppresses ROS production, counteracts free oxygen radicals, and inhibits lipid peroxidation.

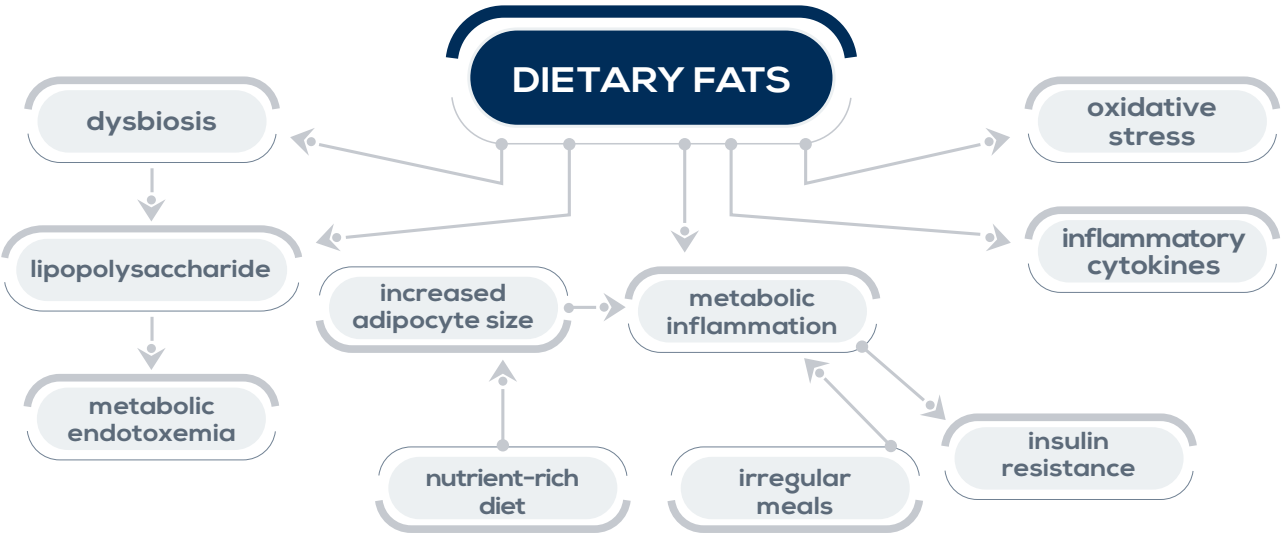
Curcumin ingestion and pharmacologic use are labeled safe by the United States FDA (Food and Drug Administration). Both curcumin and turmeric extracts are non-mutagenic and non-genotoxic; standardized powder and extract are safe for human use even at high doses of 1.5 grams a day of curcumin and for periods up to 6 months. Adverse effects, including abdominal pain, nausea, and dyspepsia, are mild and similar to the placebic treatment.

Furthermore, **resveratrol** is a polyphenol. It is found in the fruits of different blueberry species (*Vaccinium myrtillus, V. angustifolium, V. ashei,* and *V. corymbosum*), in other berries, in grapes, in peanuts, and in other plant sources; plants produce it in

response to environmental stress, to which it promotes resistance. Today, it is protective option against several lifestyle-related conditions, including inflammation and cancer.

Resveratrol downregulates the inflammatory response by inhibiting pro-inflammatory mediators and transcription factors. It is the phytochemical characterized as having the strongest activity similar to sirtuins, which are chemicals exerting an inhibitory action against COX-1. Moreover, it exerts interesting effects that are potentially useful against cancer, acting on oxidative stress and apoptosis, and influencing angiogenesis.

As with curcumin, resveratrol also inhibits Nf-kB. Moreover, it lowers the expression levels of key inflammatory factors; among others, it downregulates IL-6, IL-12 and TNF-α, and suppresses STAT3 and MMPs.



CYTOBALANCE

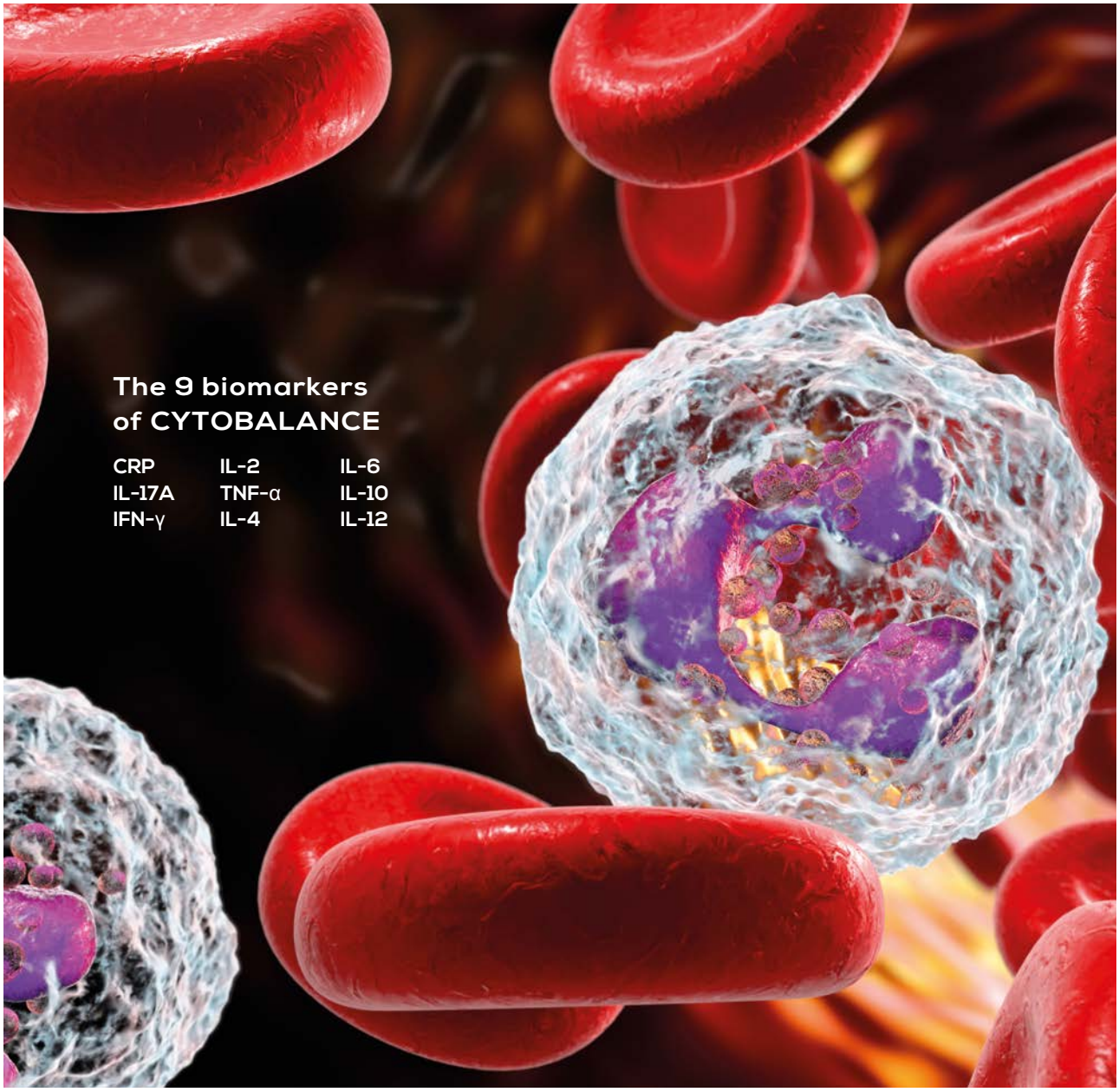
CYTOBALANCE by Bioscience Institute is aimed at controlling cancer development, that is promoting or concurring inflammation, by intercepting, monitoring, and taking action against it in healthy individuals.

The test is based on a fluorescence immunoassay allowing for significant benefits compared to traditional technologies. It is completely automatized and allows for the testing of multiple samples of multiple cytokines simultaneously. Sample size is strongly reduced (25 µl), and there is no need for manual steps increasing the risk of reduced reproducibility. Automatic control systems and a triple measurement-based quantification further increase analysis reproducibility. Cross-reactivity is eliminated by a complex microfluidic system. Finally, fluorescence-based detection enhances test sensitivity.

Among the cytokines tested are CRP, IL-17A, IFN-γ, IL-2, TNF-α, IL-4, IL-6, IL-10, IL-12**.** Moreover, **CRP** levels are tested too; testing of CRP is recognized as an easy and inexpensive tool to identify high cancer risk and people that may benefit from interventions like prophylactic therapy with anti-inflammatory drugs.

If at least one of the tested levels exceeds the attention threshold, lifestyle-based strategies (including taking dietary supplements) are suggested to reduce inflammatory mediator levels.

Maintaining inflammatory mediator levels below inflammation-associated thresholds is expected to increase genetic stability, reducing cancer risk.



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IMMUNEBALANCE
Immune System Deficiency

IMMUNEBALANCE

INTRODUCTION

Cancer formation and progression rely on a cancer cell's ability to bypass the immune response. This ability depends on genetic, epigenetic, and phenotypic changes that mask tumor cells from immune system recognition. Therefore, even if the presence of tumor-infiltrating lymphocytes was demonstrated in many tumors, cancer development is not taken under control by these immune cells, as highlighted by the tumor's growth.

Nevertheless, the immune system remains a potent inhibitor of cancer growth, and many cells that could give rise to a cancer are efficiently eliminated by immune defenses before tumor development. Several individual factors influence its activity, including sex, diet, exercise, and age – an important risk factor for cancer development.

IMMUNEBALANCE enables immune balance monitoring in healthy people, regardless of age, to detect a possible imbalanced state that can be counteracted via lifestyle-based solutions to help reduce the risk of neoplastic diseases.

AGING AND THE IMMUNE SYSTEM

Immune system stimulation and oxidative stress exposure occurring over the course of life promote a state called **inflammaging**; that is, inflammation associated with aging. This low-grade inflammation status is associated with almost all the health problems typical of aging, including cancer. It is due to chronic immune system stimulation, which induces the increase of both activated immune cells and pro-inflammatory lymphokine production, contributing to the imbalance between inflammatory and anti-inflammatory mechanisms.

Moreover, during the course of life, **oxidative stress** damages cell lipids, proteins, and nucleic acids, triggering protective mechanisms; however, as oxidative insult progresses, such protective mechanisms become less effective. The consequent accumulation of senescent, dysfunctional, and mutated cells leads to a reduction in the immune response and to an increased risk of cancer.

Besides the increased tendency for inflammation, aging also comes with **immune defense decay**. This phenomenon, known as immunosenescence, is associated with the reduction of the adaptive immune response, the enhancement of the susceptibility to infections, the increase in the production of autoantibodies (that is, antibodies directed against structures of the organism), and increased mortality. This may explain why some diseases involving the immune system (such as malignancies) develop during aging.

Actually, **the immune system is thought to play an active role in aging**, and physiological events such as the involution of thymus gland (which in the first years of life participates in immune cell maturation) support this theory. In general, the ability of constantly renewing immune system cells declines with age, and the hematopoietic tissue that produces them decreases.

The aging of the immune system compromises health span, that is the period of life free from chronic diseases and disability. In fact, immunosenescence reduces the ability to respond to new antigens (with the accumulation of memory T cells) and this inflammaging is associated with several health risks. However, aging differs from one individual to another, and the hallmarks of immunosenescence are affected by the history of individual's exposure to pathogens.

Thymus involution starts early in life and is nearly completed between 40 and 50 years old. Both innate (e.g. monocytes and Natural Killer – NK – cells, which provide a fast protection, representing body's first line of defense) and adaptive immune response (whose cells – T and B cells – respond to the inflammatory environment generated by the innate immunity, proliferating and differentiating to eliminate the health insult) progressively decrease during aging, with a significant decrease in the absolute frequencies of CD45+ Peripheral Blood Mononuclear Cells (PBMCs, including lymphocytes and monocytes). However, adaptive immunity is more extensively affected. Its effectiveness diminishes because of changes in both the quality and the quantity of the T and B cell responses. Consequentially, the reaction against newly encountered antigens becomes inadequate, and older individuals are more susceptible to the development of age-related diseases, including cancer.

AGE EFFECT ON IMMUNE SYSTEM

INFLAMMAGING

- increased activated immune cells
- increased pro-inflammatory lymphokines production
- association with age-related pathologies

REDUCED OXIDATIVE STRESS RESPONSE

- accumulation of senescent, dysfunctional, and mutated cells
- reduced immune response
- increased risk of infections, cancer, and degenerative disorders

IMMUNOSENESCENCE

- reduced adaptive and innate immune response
- enhanced infection susceptibility
- increased antibody production
- increased mortality

THYMIC INVOLUTION

- ⋁ Thymic output
- ⋀ Adoposity in thymus
- ⋁ Thymic epithelial cells
- ⋁ IL-7 production

OVERALL CHANGES

- ⋀ Tumor incidence
- ⋀ Infection susceptibility
- ⋀ Autoimmune/inflammatory reaction
- ⋁ Immune response to vaccination

INFLAMMAGING

- ⋀ Inflammatory mediators
- ⋀ IL-6, C-reactive protein
- ⋀ Tissue dysfuction

B LYMPHOCYTES

- ⋁ Naïve B cell pool
- ⋀ Memory B cell
- ⋁ IgM, IgD serum levels
- ⋀ IgG, IgA serum levels

T LYMPHOCYTES

- ⋁ Naïve T cell pool
- ⋀ Memory T cell
- ⋁ T cell receptor
- ⋀ CD8 ⋁ CD4

Adapted from: Aiello A et al. Front. Immunol. 2019 Sep 25;10:2247.

T CELLS

T cells are a type of lymphocytes generated in the thymus. Based on their co-receptor molecules, they can be distinguished in CD4+ and CD8+ cells.

Among **CD4+ lymphocytes** are T helper cells, which are the immune cells leading the fight against infections. CD4+ cells are involved in the identification and destruction of bacteria, fungi, and viruses. By releasing cytokines, they activate neighboring cells and by chemokine secretion, they recruit new immune cells.

Among **CD8+ lymphocytes** are cytotoxic T cells. These lymphocytes identify and kill cells infected by viruses and cancer cells by delivering cytotoxic granules into them. Just as CD4+ cells, CD8+ cells also produce cytokines.

Typically, a T cell response peaks in 7-15 days, after which the vast majority of antigen-specific T cells dies off, leaving a pool of memory cells. Both CD4+ and CD8+ lymphocytes can leave central-memory and effector-memory cells. The former can extensively proliferate after antigen reencounter, while the latter are characterized by limited proliferative potential but possess a rapid effector, cytolytic, function. Effector-memory cells are thus considered first responders to peripheral reinfection controlling the initial re-exposure to a pathogen, allowing central-memory cells the time to proliferate and create new effectors.

Memory cells can survive for decades, with a half-life of 8-15 years. That is why overexuberant exposure to antigens can compromise immune memory: over differentiation of T cells can limit the **immunological space** available for new memory cells. In fact, the immune system works to maintain the number of peripheral T cells at almost constant levels. Therefore, the increase in differentiated T cells reduces the space for new T cells. For example, chronic viral infections lead to continuous stimulation of T cells, increasing the risk of clonal exhaustion and over differentiation of all the viral-specific T cells into effectors.

In general, both inflammatory and immuno-modulating signals are required for proper effector and memory function. However, the overcommitment to one or the other can lead to dysfunctional immune responses.

Unfortunately, the increase in life expectancy does not always coincide with the increase in healthy, free-of-disease, years to be lived. For example, the elderly are characterized by a decrease in their ability to resist new infections. Changes in the T cell population are partially responsible for phenomena like this.

In the elderly, reduction in total T cell number has been reported, and the decreased ability to resist new infections is associated with the reduction of naïve T cells – that is, newly produced, resting cells that can be activated by an interaction with an antigen. This decrease is a consequence of both thymic involution and chronic antigenic stimulation. Moreover, elderly naïve T cells show multiple alterations, including the reduced production of IL-2 and the diminished ability to differentiate into effector cells. Finally, senescent cells' reduced sensitivity to damaged-induced apoptosis reduces the immunological space; thus, depleted CD4+ cells cannot be replaced with either CD4+ or CD8+ cells. In fact, aging-associated reduced sensitivity to damaged-induced apoptosis promotes the accumulation of dysfunctional cells, CD8+ cells, and memory cells, reducing the space for other immune cells, in particular CD4+ cells. This is why the accumulation of these cells increases the risk of both infections and neoplastic or degenerative disorders even without significant changes in the total CD3+ (T) cell level.

Factors affecting the elderly can progressively influence the CD4+/CD8+ ratio, a parameter telling how strong the immune system is. CD4+/CD8+ ratio is considered a component of the increased risk of death. In very old individuals the combination of high CD8+ and low CD4+ percentages and poor T cell proliferation in peripheral blood lymphocytes is associated with higher mortality. Moreover, the mortality rate above the age of 60 is significantly increased when CD4+/CD8+ ratio is inverted. That is why CD4+/CD8+ inversion evaluation is crucial for individuals suspected of having a compromised immunity.

CD4+/CD8+ ratio is high in neonates and declines into adult values by 4 years of age. **Its inversion is a feature of the so-called immune risk phenotype** (IRP), a condition typical of the elderly characterized by a memory-effector cell increase. Interestingly, IRP is independent of their overall health status, not being exclusive to frail individuals. Moreover, there is a significant lowering of T cells CD3+, CD4+ and CD8+ cells across the adult lifespan, and the prevalence of CD4+/CD8+ ratio inversion increases with aging, shifting from about 8% in 20–59-year-old individuals to 16% in 60-94-year-old individuals. Finally, CD4+/CD8+ inversion was reported to be significantly more frequent among men. This phenomenon was suggested to contribute to the differences in longevity between sexes.

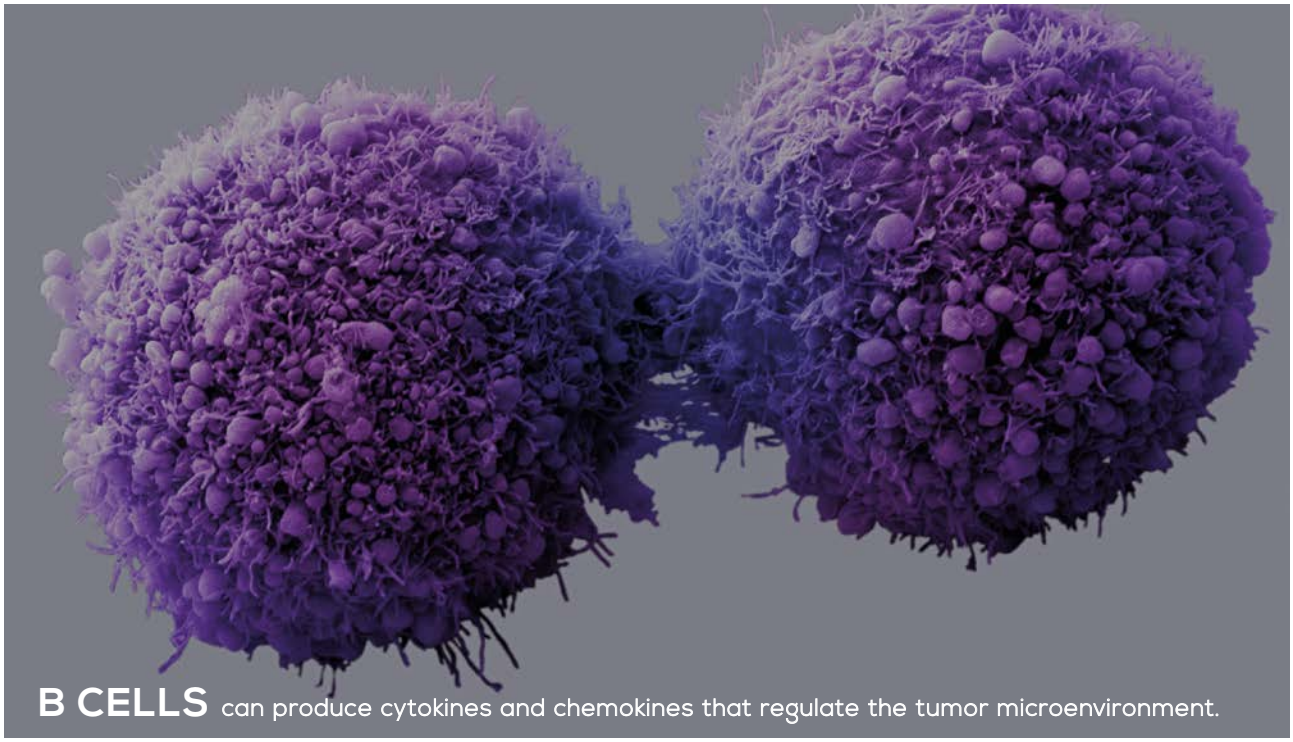
CD4+/CD8+ inversion might represent a step toward a progressive CD4+ lymphocytopenia, a rare condition in aged people. In this case, factors affecting the CD4+ population could be the cause of the inversion. However, aging-associated CD8+ cell changes are more important. In particular, healthy elderly clonal expansion has been reported and has been associated with a CD28- subpopulation, lacking in proliferative responses to T cell receptor stimuli.

Moreover, the CD4+/CD8+ balance may be altered by some conditions, particularly viral infections. Cytomegalovirus (CMV) positivity, a leading cause of immune system senescence, is closely related to the inversion of the CD4+/CD8+ ratio. CMV infection, that persists lifelong after the first exposure, chronically stimulates CD8+ cells, which expand and demonstrate a memory-effector phenotype. CD4+ cells decrease in a compensatory way. In the IRP, marked seropositivity for cytomegalovirus is associated with reversal of CD4+/

CD8+ ratio, the increase in CD8+CD28- memory-effector cells and in pro-inflammatory cytokines such as IL-6, and the reduction of B cells (CD19+). This condition is predictive of the development of cognitive deficits, and of mortality rate over the next 4 years in 58% of cases. Even if a significant number of adults are positive for CMV, only a minor part of them is aware of its seropositivity; as a consequence, they are unaware of their possibly compromised immune defenses.

Other conditions associated with CD4+/CD8+ inversion are transplantation, hemophilia treatment, acute illness, and malnutrition. In many cases, T cell subpopulations are only temporarily affected; however, CD4+/CD8+ ratio can remain altered in the long term. In general, both microbial agents and environmental stressors can initiate T cell changes; an immune system lacking the required resilience might foster a progressive change in the CD4+/CD8+ ratio toward unhealthy values.

In the elderly, CD4+/CD8+ ratio values greater than 2 have been reported. It must be noted that, besides strong immunity, such values can be associated with specific pathologies, such as infections and blood cancers. Moreover, as opposed to the mean, the median elderly CD4+/CD8+ ratio tends towards lower values, indicating a tendency for inversion.



B CELLS can produce cytokines and chemokines that regulate the tumor microenvironment.

B cells are immune cells responsible for the production of antibodies, antigen presentation and cytokine secretion. They are released as immature cells by the bone marrow, and their total number, as well as the composition of the B cell population circulating in peripheral blood, dynamically changes during the course of life.

Immediately after birth total B cell count increases 2-fold. This increase is due to an active production and release of B cells by the bone marrow, that reaches a maximum between 0 and 12 months of age. Then B cell count remains high until 2 years old, when a gradual decrease starts, leading to approximately a 6.5-fold decrease in adulthood.

Starting from 2 months of life, memory B cells increase too. Their number remains high until the age of 5 years old, when it starts decreasing to adult-like values.

In adulthood, total B cell number remains relatively stable until aging. However, **aging is associated with the reduction of B cell production** by the bone marrow. Recent analyses suggest that this age-related decline is due to the increased production of inflammatory cytokines that can inhibit their synthesis, such as IL-1, IL-6 and TNF-α. In young people, short-term inflammation may stimulate the production of myeloid cells, such as monocytes, that help to promptly respond to infections; however, the chronic inflammation associated with aging may translate into a continuous inhibition of B cell production.

Moreover, memory B cells and new plasma cells (that is, the more advanced differentiation step of mature B cells, able to secrete large amounts of antibodies) gradually decrease after 60 years of age.

MONOCYTES

Monocytes are white blood cells developing in the bone marrow that are involved in both the innate immune response, and processes such as tissue repair. They circulate in the blood, migrating to sites of injury or infection after the engagement of chemokine and pathogen recognition receptors. They can perform pro-inflammatory and pro-resolving functions, take up cells and toxic molecules, and differentiate into inflammatory dendritic cells or macrophages.

Two major monocyte subsets in the peripheral blood can be distinguished based on the differential expression of two molecules: CD14 and CD16. **CD14++CD16-** cells are known as "classical monocytes", whereas **CD14+CD16+** cells are known as "non-classical monocytes". A third subset of monocytes is represented by **CD14++CD16+** cells, or "intermediate monocytes".

CD14++CD16- cells represent up to 95% of monocytes in healthy individuals. They are characterized by a high phagocytic capacity, innate sensing/immune responses, and migration, and can be somewhat more efficient than CD14++CD16+ monocytes in producing reactive oxygen species (ROS) and constraining fungi. They can differentiate into monocyte-derived macrophages and dendritic cells, which are immune cells playing a pivotal role in the development and resolution of tissue inflammation.

Compared to them, CD14++CD16+ cells display similar phagocytosis potential, but lower adhesion capacity, and greater class II molecule expression and IL-2 production. They are well-suited for antigen presentation, cytokine production (TNF-α, IL-1β and IL-6), apoptosis regulation and differentiation. Their blood count is expanded in cases of systemic infection, so they must play an important role in the rapid response to pathogens.

CD14+CD16+ monocytes are instead associated with wound healing and antiviral responses and promote neutrophil adhesion at the endothelial interface via TNF-α production. However, they do not produce the same levels of pro-inflammatory cytokines as classical monocytes. Moreover, compared to classical CD14++CD16- cells, non-classical CD14+CD16+ monocytes express lower levels of chemokine receptors involved in monocyte migration.

CD16+ subsets correlate with chronic inflammation-associated pathologies. Their absolute number and the relative contribution to the circulating monocyte pool increase also during aging. In fact, in healthy adults, total monocyte levels do not change with age, but after the age of 50 **there is a shift from classical CD14++CD16- monocytes to CD14+CD16+ cells**. This could represent a physiological trick to preserve the overall functionality of the monocyte pool, but it does not necessarily correspond to enhanced monocyte availability and functionality in aging.

Indeed, after the age of 50 the functionality of CD14+CD16+ monocytes seems to decrease. They express lower levels of activation markers and display a potentially reduced capacity to migrate to inflammatory sites and to the spleen. Moreover, the half-life of monocyte-derived tissue macrophages is lower too.

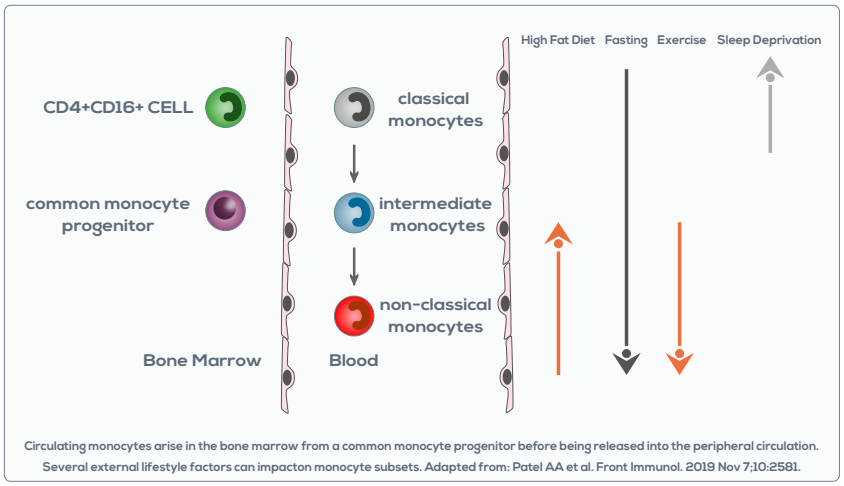
Finally, **CD14+CD16+ monocytes that are expanded after the age of 50 are generally believed to have the capacity to produce higher levels of pro-inflammatory cytokines.** Specifically, advanced-age, frail elderly demonstrate a higher production of TNF-α, and to a lesser extent IL-8. Pro-inflammatory cytokine production is constitutively higher, suggesting a predisposition to chronic disease development.

Looking at the differences between adults (19-59 years), seniors (61-76 years), and the advanced-aged, frail elderly (81-100 years), the latter showed a significant reduction in CD14++CD16- and CD14+CD16+ monocytes, and an increase in CD14++CD16+ cells.

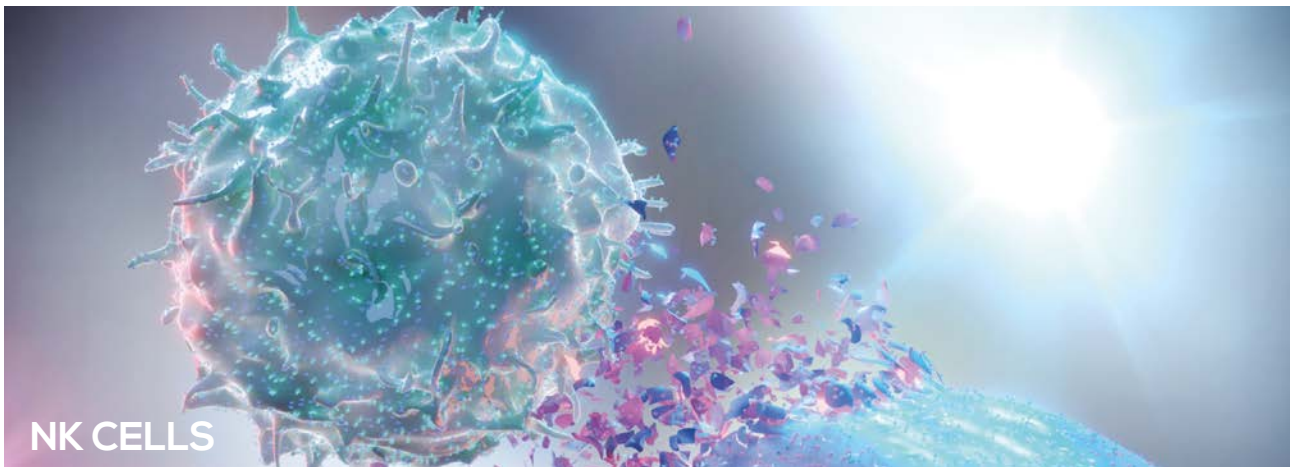
Overall, data in the scientific literature indicate a decrease in CD14++CD16- monocytes and an increase in CD16+ subpopulations starting from 61 years of age. CD14+CD16+ cells seem to increase after the age of 50, but their functionality is compromised; starting from the age of 81, frailty is associated with their reduction, whereas CD14++CD16+ monocytes increase.

CD16+ cells, specifically **non-classical monocytes, seem to account for the increase in plasma TNF-α levels and inflammatory conditions in aged individuals.** Monocyte frequency, phenotype, and functional changes observed in the advanced-age, frail elderly correlate with chronic diseases associated with a chronic inflammatory state.

Interestingly, the reversal of this CD16+ cell increase seems to be possible. In obese individuals, for example, both dietary intervention and gastric bypass surgery result in the decrease of these subsets of monocytes.



Circulating monocytes arise in the bone marrow from a common monocyte progenitor before being released into the peripheral circulation. Several external lifestyle factors can impacton monocyte subsets. Adapted from: Patel AA et al. Front Immunol. 2019 Nov 7;10:2581.



NK CELLS

Natural Killer (NK) cells are versatile lymphocytes involved in both innate and adaptive immunity that can destroy virus-infected and tumor cells. Their cytotoxicity relies on the fine balance between activating and inhibiting surface receptors. They can secrete cytokines, regulate dendritic cell maturation, and act as antigen presenting cells.

NK cells can be divided in subsets based on CD56 and CD16 expression. **$CD56^{dim}CD16+$** cells are mature cytotoxic cells with a poor capacity to proliferate in response to cytokines. They represent up to 90% of NK cells circulating in peripheral blood cells, and directly kill target cells via exocytosis of granules, activation of cell death, or antibody-dependent cytotoxicity. After direct contact with target cells, **$CD56^{dim}CD16+$ cells** secrete cytokines such as IFN-γ.

$CD56^{bright}$ cells are less mature and are concentrated mostly in lymph nodes. They produce and are responsive to cytokines; in particular, **$CD56^{bright}CD16-$ cells** produce high levels of cytokines and chemokines in response to IL-2, IL-12, and IL-18.

Although there is some conflicting evidence, several data suggest that **during aging** absolute peripheral blood NK cell numbers tend to increase. However, starting from the age of 50 the production of new NK cells is reduced, indicating **a high proportion of "old" NK cells** in the elderly. **$CD56^{bright}$ cells decrease**, probably because of the age-associated changes in the hematopoietic stem cells in the bone marrow. Consequently, in the elderly cytokine and chemokine production by NK cells is impaired. Instead, the production of IFN-γ increases, probably as a compensatory mechanism to maintain immunoregulatory role of $CD56^{bright}$.

Meanwhile, **the subset of CD56-CD16+ NK cells increases**. These cells are characterized by **low replicative capacities and reduced cytokine production.** CD57+$CD56^{dim}$CD16+ cells increase too. This subpopulation, characterized by high cytotoxic capacity and reduced sensitivity to cytokines and replicative potential, is absent at birth and increases with age. However, CD57 seems to be a marker of NK cell expansion in response to CMV infection; in CMV-positive individuals, it accumulates over time to maintain NK cell homeostasis. However, the redistribution of NK cell subsets, with $CD56^{bright}$ decreasing and CD56-CD16+ cells increasing, is associated with aging but not with CMV infection.

In general, **low NK cytotoxicity is associated with an increase in morbidity** (e.g. infections and mechanisms of atherosclerotic and neurodegenerative diseases) **and mortality.** On the contrary, high NK function is associated with longevity and good health.

NK cell subsets				
	$CD56^{bright}$ CD16-	$CD56^{bright}$ CD16+	$CD56^{dim}$ CD16+	CD56- CD16+
CMV	=	=	=	=
AGE	⋁	⋁	= OR ⋀	⋀

Adapted from: Solana R et al. Curr Opin Immunol. 2014 Aug; 29:56:61

VITAMIN D AND THE IMMUNE SYSTEM

Immune system efficiency depends also on vitamin D status. This peculiar micronutrient (in effect, a prohormone) is a potent immunomodulator. Before the advent of efficient antibiotic therapies, it was used to treat serious infections, such as tuberculosis. Today, **the association between its deficiency and the increased susceptibility to infection** (both respiratory and oral, gastrointestinal, genitourinary tract, and ocular) is well-known. Moreover, the link between inadequate vitamin D status and the prevalence of autoimmune disorders has been unveiled too. In general, avoidance of vitamin D deficiency is associated with better immune system functioning.

In humans, it is synthesized in the skin by the action of ultraviolet (UV) rays upon its precursor, 7-dehydrocholesterol. Its active form is then obtained by hydroxylation taking place in the liver and subsequently in the kidney. In the circulation, vitamin D and its metabolites are bound to the vitamin D-Binding Protein (DBP), which functions as an inflammation and immune response regulator, too.

Immune cells possess the necessary machinery to synthesize its active form too. Moreover, they express the vitamin D receptor (VDR). Thus, the immune system can both produce and respond to vitamin D, and vitamin D can act on immune cells both in a paracrine and autocrine manner. It exerts a complex effect, promoting a more tolerogenic immune status.

First, **vitamin D plays an important role in the innate response** to microbes. Its antimicrobial role is mediated by monocytes. In these cells, vitamin D exerts an anti-oxidative effect. Moreover, it inhibits the production of cytokines such as IL-1, IL-6, IL-8, IL-12, and TNF-α. Finally, recent studies suggest an epigenetic effect during antigen encounter and differentiation.

In macrophages, the activation of toll-like receptors (TLRs) by microbial components leads to the increase of the expression of both the vitamin D synthesizing enzyme and the VDR. This results in the production of antimicrobial proteins. Moreover, the active form of vitamin D exerts an anti-inflammatory action on these immune cells. It increases IL-10 and decreases inflammatory stimuli, such as IL-1β, IL-6, TNF-α, and cyclooxygenase-2 (COX-2). VDR expression levels are reduced compared to monocytes. The enzyme responsible for vitamin D synthesis is dependent on 25-hydroxyvitamin D3 (25-(OH)D3) produced in the liver, and may be induced by cytokines such as IFN-γ, IL-1, or TNF-α.

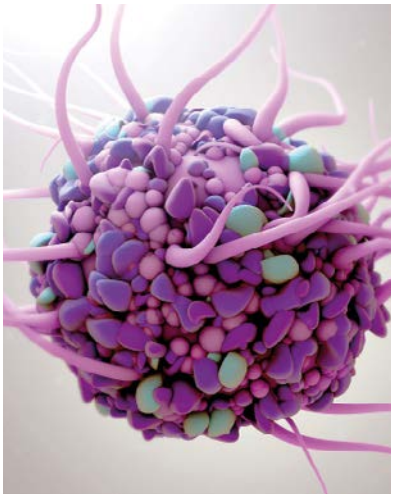
The active form vitamin D (1,25-(OH) D3) induces changes in dendritic cell morphology, cytokine production, and surface markers, promoting a less mature and more tolerogenic phenotype. IL-6 and IL-12 are reduced, whereas IL-10 is increased, together with molecules that regulate T cell activity, such as TNF.

Finally, vitamin D regulates NK cells and neutrophils as well. In particular, by modulating the activity of neutrophils, this vitamin helps to minimize damage by pathogens and reduce the risk of autoimmunity.

Vitamin D also affects adaptive immunity. First, it seems to directly act on B cells, inducing the apoptosis of activated lymphocytes and impeding the generation of plasma cells (that is, antibody-producing cells) and memory B lymphocytes. Moreover, it upregulates B cell IL-10 production.

Secondly, acting on antigen presenting cells (that is, immune cells that collect antigens and communicate with lymphocytes to orchestrate the adaptive immune response), vitamin D influences T cell activity. Specifically, it modulates T cell responses reducing T cell autoreactive proliferation, inducing the apoptosis of autoreactive T cells, and increasing T lymphocytes that downregulate the immune response.

Vitamin D can also directly influence T cell activity, promoting the reduction of inflammatory cytokines (IL-2, IL-9, IL-17, IL-21, and IFN-γ) and the increase of anti-inflammatory cytokines (such as IL-10). Its effect is dependent on the state of activation of T cells; in fact, T



cell VDR concentration increases upon their activation.

Unfortunately, **up to 40% of adults suffer from low serum vitamin D levels.** This widespread vitamin D deficiency is caused by a combination of a scarcity of vitamin D-rich foods and poor UV-synthesis.

Salmon, rainbow trout, swordfish, mackerel, herrings, eggs, tuna, milk, and bovine liver are the richest sources in the diet. However, largest dose of this peculiar nutrient required to fulfill daily organism requirements is synthesized in the skin by the action of solar UV-rays.

People spend less time in the open air than in the past, and they need to protect their skin from cancer risk by blocking UV-rays with adequate sunscreen. Moreover, latitude, season, and skin pigmentation influence skin vitamin D synthesis. All these factors can limit their exposure to adequate UV-rays.

Aging comes with an even greater risk of severe vitamin D deficiency. In a study involving 75-year and older men and women, French and Canadian researchers found the recommended vitamin D level only in 15% of the participants and classified more than a quarter of them (27%) as affected by a “very severe vitamin D deficiency”. Severe, moderate, and minor vitamin D deficiencies were found, respectively, in 16%, 27%, and 15% of the participants.

No significant difference was observed between age subgroups, suggesting that all elderly people might be at a higher risk of deficiency.
Other studies showed even smaller percentages of normal vitamin D concentration in the elderly (6-7.5%). In general, available data demonstrate that vitamin D deficiency in the elderly is a public health problem. Reasons behind it include the already cited low sun exposure and scarce availability of dietary sources, as well as the lack of physical activity, and the reported decrease in skin vitamin D synthesis.
This deficiency is traditionally associated with bone fractures, but not exclusively. In fact, low serum vitamin D level was reported to be associated with frailty syndrome and as previously stated, with several infections. Vitamin D status correlates with cardiovascular disorders, diabetic retinopathy, migraines, and cancer (in particular bladder carcinoma and colorectal cancer). Furthermore, vitamin D supplementation has been associated with a significant improvement in senile dementia-associated cognitive performance decline and type 2 diabetes fasting blood glucose. In summary, there are plenty of reasons to warrant the organism adequate vitamin D levels in adulthood.

The nutritional vitamin D status is reflected by 25-(OH)D levels. To warrant immune system efficiency, all deficiencies must be corrected. Supplementation represents an effective strategy. For example, both vitamin D2 and vitamin D3 have been associated with protection against acute respiratory tract infections in 25-(OH)D3 deficient adults, especially with a daily or weekly supplementation.

IMMUNEBALANCE

Monitoring immune system balance allows for intercepting immune function's aging and makes counteracting it to reach old age without disability possible. The adequate modulation of immune responses and the counterbalance of a pro-inflammatory cytokine profile with an anti-inflammatory profile can help reduce age-related degenerative, inflammatory, and neoplastic diseases.
IMMUNEBALANCE by Bioscience Institute enables immune balance monitoring in healthy people, regardless of age, by the simultaneous analysis of total lymphocytes, T and B cells, CD4+/CD8+ T cell ratio, CD14 and CD16 monocyte expression, NK cells, and vitamin D levels. It intercepts deviations from healthy values before the onset of health problems. The eventually detected imbalanced state can be counteracted via lifestyle-based solutions, such as the use of food supplements specifically studied to promote immune system functioning.

PARAMETER	DISEQUILIBRIUM
Total lymphocytes	⋁
T cells	⋁
CD4+ T cells	⋁
CD8+ T cells	⋁ or ⋀
CD4+/CD8+	⋁ or ⋀
B Cells	⋁
Monocytes	
classical/intermediate	⋁
intermediate/non-classical	⋀
NK cells	⋀
$CD56^{bright}$	⋁
CD56-CD16+	⋀
Vitamin D	
25-(OH)D	< 30 ng/ml (insufficiency) < 20 ng/ml (deficiency)

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MICROBALANCE
Gut bacterial imbalance

MICROBALANCE

INTRODUCTION

The human organism is not a sterile environment: both skin and internal tissues house a large variety of microorganisms peacefully living with their host, contributing to its health status. This set of microbes (bacteria, fungi, protozoan, and viruses) is called “microbiota” and comprises 100 trillion (billions of billions) bacteria, whose genetic material is called “microbiome”.

The majority of these microbes lives in the gut, where they form the so-called gut microbiota, a community whose genomes – the gut microbiome – encode a number of genes 100-fold greater than human gene number. In total, gut microbiota comprises more than 50 bacteria phyla – in particular anaerobes such as Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria – and more than 15 thousand kinds of bacteria, and weight about 1 kg. The colon (the terminal part of the bowel) is the most densely populated region; here, each gram of the intestinal content comprises 100 billion bacteria.

The idea of microbiota playing a role in human health promotion arises from studies on the function of lactobacilli as vaginal ecosystem gatekeepers and the discovery of the association between fermented milk product consumption and prolonged life. Today, it is known that health is shaped by microbiota robustness, and that its capability to resist and recover from change acts as a shield preventing pathogenic invasions of the skin, mouth, and gut. Moreover, gut microbiota are critical for immune system development and for the establishment of immune tolerance.

Among diseases associated with microbiota is cancer. Besides influencing anticancer treatments, microbes living in the organism and on its surfaces can act as cancer drivers or exert a protective effect via several mechanisms. That is why microbiota analysis is a powerful tool in cancer risk interception and monitoring.

MICROBIOTA AND CANCER DEVELOPMENT

The microbiota exerts both local and systemic effects on cancer. In some cases, its composition promotes the development of the disease. However, some bacteria exert a protective effect, reducing the risk of cancer.

Stomach, liver, and colorectal cancers stand as examples of the association between unfavorable microbiota features and cancer. In fact, stomach cancer development depends on the bacterium *Helicobacter pylori,* whereas the microbiota of colorectal cancer patients is characterized by increased *Prevotella*, and liver cancer has been linked to microbiota-derived bile acids that damage liver cell DNA. But the list of cancers in which the microbiota has been implicated in the carcinogenic process is longer; among others are bladder carcinoma, lymphoma, prostate cancer, breast carcinoma, sarcoma, pancreatic cancer, and ovarian cancer.

In some cases, single bacterial strains are responsible for driving the development of the disease, such as *Helicobacter pylori* in stomach cancer, *Escherichia coli* in colorectal carcinoma, and *Salmonella enterica typhi* in bladder carcinoma. Also, carcinogenesis can be locally modulated by groups of bacteria, which contribute to cancer development by interacting with the altered environment.

MICROBIOTA-ASSOCIATED CANCERS

stomach cancer	prostate cancer	hepatocellular carcinoma	sarcoma
colorectal cancer	breast cancer		ovarian cancer
bladder cancer	pancreatic cancer	lymphoma	

In other cases, cancer is driven by overall microbiota characteristics than by the presence of one species or one group of microbes, such as changes in bacterial density near mucous membranes.

The vaginal microbiota has been linked to gynecological cancer, whereas organs with no known microbiota, such as liver, can be affected because of anatomical links with the gut allowing exposure to microbe-specific molecules (the so-called microorganism-associated molecular patterns, or MAMPs) and bacterial metabolites.

Among mechanisms underlying the association between the microbiota and cancer development is the relationship between bacteria, **inflammatory cytokines**, and the **immune system**.

Furthermore, some bacteria can affect cancer development by deregulating signals or pathways. For example, *Helicobacter hepaticus* promotes liver cancer by activation of NF-kB regulated networks.

Other bacteria directly affect carcinogenesis by producing virulence factors (such as toxins) that modulate inflammation in the cancer microenvironment, influence **genomic stability** of host cells, or epigenetically regulate host gene expression. For example, in colorectal cancer colibactin-synthesizing *Escherichia coli* is associated with DNA damage.

Moreover, microbiota can interact with food and inflammation-related host metabolites that increase the growth of potential pathogens and the risk of DNA damage. It can, for example, enhance the production of secondary bile acids hypothesized to modify the mucosal barrier, allowing for antigen penetration and increasing inflammation and the production of factors needed for cancer development. Also, it can metabolize red meat and high processed food, giving rise to molecules believed to damage DNA, such as hydrogen sulfide.

Finally, the correlation between **obesity**, a known risk factor for cancer, and gut microbiota is well documented, and **aging**, a well-known cancer driving factor, is associated with gut microbiota alteration as well.

SOME BACTERIA DIRECTLY INVOLVED IN CANCER DEVELOPMENT

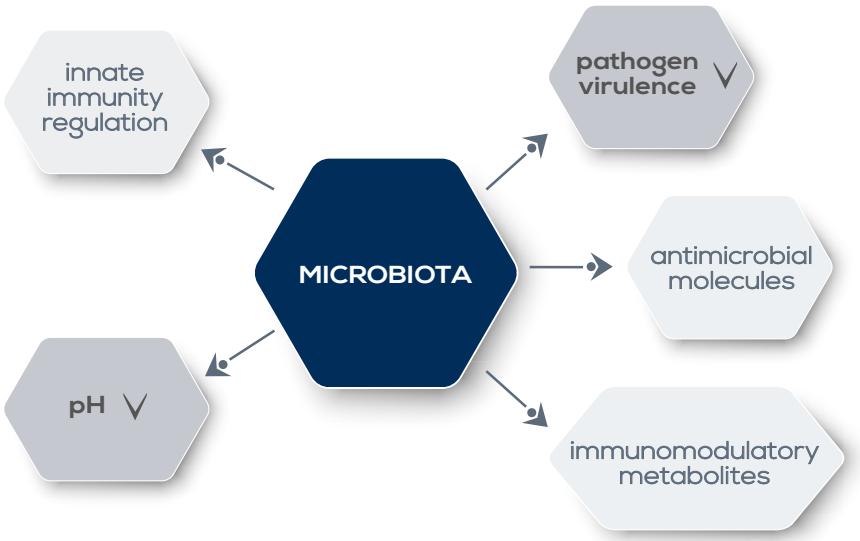
Helicobacter pylori	Esophageal adenocarcinoma
	Gastric adenocarcinoma
	Gastric lymphoma
	Colorectal cancer
Salmonella enterica typhi	Bladder cancer
Fusobacterium nucleatum	Colorectal adenoma
Helicobacter hepaticus	Liver cancer
Bacteroides fragilis	Colorectal cancer

GUT MICROBIOTA AND THE IMMUNE SYSTEM

Gut microbiota plays a fundamental role in the induction, education, and function of the immune system, establishing an alliance with its host's defense weapons interweaving innate and adaptive immunity to select, calibrate, and terminate responses. The training of this system during infancy and childhood leads to the establishment of a durable relationship. The immune system continuously works to control this relationship, and the microbiota constantly reinforces the so-called "**barrier immunity**", that is the mechanisms that allow its containment.

These mechanisms consist of strategies to minimize the contact between microorganisms and bowel epithelial cells. First, intestinal cells produce a mucus layer that limits contact between the microbiota and host tissues and prevents microbial translocation in the bloodstream. Moreover, intestinal cells produce antimicrobial molecules too. Third, immune cells in the intestines produce antibodies (IgA) that are specific for microbiota antigens, and white blood cells in the intestinal wall (namely, macrophages) rapidly eliminate microbes translocating across the epithelial cell barrier.

The body's recognition of commensal microbiota is a critical step to be taken early on in life, during the preweaning period. A certain degree of microbiota recognition is a common occurrence, in most cases it is not associated with a pathogenic response. However, gut dysbiosis - that is, alterations of **gut microbiota** - is associated with a number of health problems. Overall, allergic sensitization, eczema, and asthma are associated with a lower relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae* and a greater relative abundance of *Bacteroidaceae, Clostridiaceae*, and *Enterobacteriaceae*. Meanwhile, a greater microbiota diversity promotes the development of a healthy immune system, reducing the risk of asthma and allergic diseases.



However, gut microbiota's role in human health goes far beyond immune system training: it is fundamental also for the immune system's functioning under a steady state and inflammatory conditions, and for tuning the inflammation generated by cells involved in the control of pathogens in the bowel, protecting host tissues from damage.

The mechanisms by which the microbiota shapes immunity are not yet completely understood. Commensal bacteria-derived signals could influence the expression of genes involved in **innate responses**, enabling a basal level of host-defense factors and a rapid response upon pathogens. What is known is that the microbiota directly and dynamically interacts with pathogens. They compete for the **same ecological niche**; moreover, microbiota metabolites that modulate the activity of the immune system (such as short-chain fatty acids, **SCFAs**) can also downregulate the expression of pathogen virulence genes. Moreover, the microbiota promotes the establishment of a **hostile environment for pathogens**, for example by lowering local pH or by producing antimicrobial molecules.

Alterations in gut microbiota can affect both local immune responses as well as immunity and inflammation in other organs, even if they are distant from the intestine. For example, a reduction of "good" gut bacteria via antibiotic treatment reduces the immune system's response against intranasal influenza infection. This control on systemic immunity has profound consequences also in the context of therapy.

Gut microbiota shapes and modulates the host immune system and plays a pivotal role in **digestion and absorption of dietary components**. For example, it can extract nutrients and energy from food fibers that would otherwise be lost because humans lack enzymes required to harvest them. Metabolites produced during such reactions can influence immune system functioning.

Finally, gut microbiota participates in the **detoxification of** potentially dangerous substances, including **carcinogens**.

GUT MICROBIOTA AND INFLAMMATION

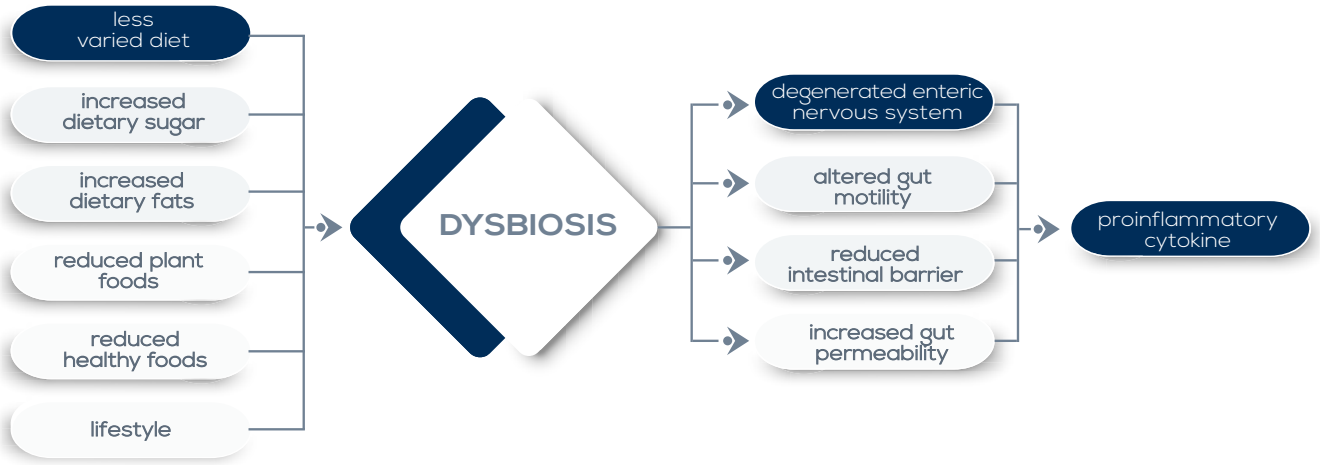
Notably, intestinal cancer is inextricably linked to gut inflammation, and Crohn's Disease, an inflammatory bowel disease, is associated with colorectal cancer development. Several mechanisms are involved in this relationship, from innate and adaptive immune cell migration to endocrine and neural pathways, translocation of bacteria or bacterial products and toxins, and systemic inflammation and oxidative stress modulation.

Gut microbiota seems to play an important role in the development of these and other inflammation-associated diseases and conditions. The outgrowth of opportunistic bacteria can drive an increase in inflammation, and the loss of benign bacteria reduces immunoregulation. Dominance of bacteria characterized by enhanced invasiveness and inflammatory properties (in particular γ-proteobacteria) can **directly exacerbate inflammation and tissue damage**, and mechanisms such as their capability to thrive on metabolites derived from inflammatory settings could contribute to their proliferation.

Moreover, external factors such as the overuse of antibiotics, changes in diet, and the elimination of chronic parasitic infections may select for microbiota lacking the resilience required for the establishment of a balanced microbiota-host interaction in which stimulatory and regulatory signals allow for immunity development without compromising the tolerance to innocuous antigens. Such a selection is now believed to contribute to the increase in chronic inflammatory and autoimmune disorders seen in westernized countries.

In fact, many inflammatory diseases are associated with significant alterations in the resident microbiota, shifting from a healthy to a diseased state. Susceptibility to some diseases could be at least in part due to the presence of specific bacteria particularly adept at surviving in, and contributing to, inflammation. Evidence of this phenomenon comes both from mice and human studies.

In particular, the development of Inflammatory Bowel Diseases (IBDs, namely Crohn's disease – CD – and ulcerative colitis – UC) is believed to be the result of a combination of genetic factors, the immune system's activity, and environmental factors – including the microbiota. Both CD and UC are associated with a reduced complexity of the microbiota and with a dysbiosis characterized by the outgrowth of proteobacteria (in particular, the *Enterobacteriaceae family*). Moreover, in CD patients, commensal microbiota that are intrinsically inflammatory (such as *Escherichia coli, Yersinia* and *Clostridium difficile*) are much more common than in healthy individuals, and there is an increase in serum antibodies against the microbiota. Currently, it is thought that IBDs are caused or exacerbated by a loop where host mutations leading to dysregulated immune responses in the gut drive the outgrowth of bacteria that promote more inflammation. In parallel, the loss of bacteria producing SCFAs (that have been shown to limit gastrointestinal inflammatory processes) may contribute to gut inflammation. In particular, *Clostridia* – a class of *Firmicutes* that directly induces immune cells opposing colitis induction and ferments dietary fiber to SCFAs – are reduced in IBDs patients.



GUT MICROBIOTA AND GENOMIC INSTABILITY

Gut microbiota can influence genomic stability of the host in both direct and indirect ways. Colorectal cancer stands as an example of this phenomenon.

There are two **indirect mechanisms**. First, gut microbes can elicit a chronic inflammatory response, which may promote the release of DNA-damaging reactive oxygen species (ROS) and reactive nitrogen species (RNS). Dysbiotic microbiotas constantly provide antigens, such as components of the bacterial cell, which stimulate the host immune system, inducing chronic inflammation.

Second, gut microbes can produce secondary metabolites (such as SCFAs, hydrogen sulfide, and secondary bile acids) that can impact the host genome. Specifically, SCFAs exert a protective role by suppressing inflammation, whereas hydrogen sulfide, secondary bile acids, and other molecules cause DNA damage, exerting a pro-carcinogenic effect.

Direct mechanisms include the production of ROS and RNS or toxins by bacteria. For example, in *in vitro* studies, colibactin produced by pks+ *Escherichia coli* was associated with the induction of DNA double-strand breaks and chromosomal aberration. Working synergistically with enterotoxigenic *Bacteroides fragilis,* pks+ *E.* coli increases DNA damage, inflammation, and tumor formation in a mouse model of colorectal cancer. Other bacterial toxins that can directly damage DNA and modulate tumorigenesis are cytolethal distending toxin and cytotoxic necrotizing factor 1. Besides *E. coli* and *B. fragilis, Shigella dysenteriae, Actinobacillus actinomycetemcomitans, Campylobacter, Helicobacter, Salmonella typhi*, and *H. ducreyi* have been suggested to release toxins that can cause DNA damage, cell cycle arrest, or apoptosis. Also, enteropathogenic *E. coli* is associated with the depletion of host proteins needed for DNA repair, leading to an increased frequency of mutations.

Furthermore, chromosomal instability seems to be induced by a microbial-macrophage interaction. Specifically, by influencing macrophages, *Enterococcus faecalis* was associated with chromosomal instability induction and cancerogenic DNA mutations in cancer-driving genes.

GUT MICROBIOTA AND OBESITY

Both obesity and its comorbidities are associated with changes in gut microbiota. In general, obesity is associated with a **lesser diversity and richness of gut microbiota**, and in obese people the ratio of *Firmicutes* to *Bacteroidetes* seems to be higher. Moreover, some studies highlighted a decrease of *Methanobrevibacter smithii* in obese individuals and a strong association between obesity and *Blautia hydrogenotorophica, Coprococcus catus, Eubacterium ventriosum, Ruminococcus bromii, Ruminococcus obeum,* and *Lactobacillus reuteri,* as opposed to a larger proportion of *Bacteroides faecichinchillae, Bacteroides thetaiotaomicron, Blautia wexlerae, Clostridium boltae, Flavonifractor plautii,* and *Bifidobacterium* and *Lactobacillus* species in lean people.

Low bacterial richness is also associated with obesity-related conditions, in particular insulin resistance and dyslipidemia.

Insulin resistance has been associated with *Lactobacillus* increase and *Clostridium* reduction, and in type II diabetes, the disease can be exacerbated by chronic inflammation driven by complex interactions between the immune system and gut microbiota. SCFA-producing *Clostridia* strains are reduced whereas *E. coli* increases, and blood translocation of bacterial products may rise – as suggested by increased serum lipopolysaccharide (LPS) levels.

An in-depth study of the link between obesity and gut microbiota has been conducted mainly in animal models, but several data suggest that similar relationships are present in humans too.

Among the proposed mechanisms is the ability of gut microbes to regulate energy intake by fermenting dietary fibers. Due to the higher presence of enzymes for complex carbohydrate degradation and fermentation, obesity-associated gut microbiota is characterized by an increased capability to harvest energy from the diet. Fermentation-derived SCFAs can induce lipogenesis, increase triglycerides stores, inhibit lipolysis, and encourage adipocyte differentiation. Moreover, feces from obese individuals are characterized by higher SCFAs levels and reduced residual food calories than feces of lean individuals.

Moreover, obesity-associated gut microbiota impairs triglyceride metabolism and promotes fat storage by stimulating gene reprogramming in the colon. It also inhibits the enzyme adenosine monophosphate kinase (AMPk), resulting in decreased fatty acid oxidation and, as a consequence, increased fat accumulation.

Gut microbiota LPS can trigger systemic inflammation too, contributing to metabolic disturbances associated with obesity. Dysbiosis increases gut permeability to bacterial products such as LPS and ethanol.

Finally, obesity-associated gut microbiota changes hormones and other bioactive molecules involved in regulating food intake released by intestinal endocrine cells, alters gut-brain communications via molecules such as LPS, gut peptides and hormones, SCFAs, and lactate, and seems to contribute to metabolic disorders through an axis of communication with adipose tissue. For example, LPS triggers insulin resistance in adipose tissue, lactate contributes to postprandial satiety, and gut bacteria-derived serotonin and γ-aminobutyric acid affect the central control of appetite.

DIET AND THE MICROBIOTA: EXAMPLES

vitamin D	⋁ Lipopolysaccharides ⋁ *Coprococcus, Bifidobacterium* ⋀ *Prevotella*
emulsifiers	Alteration of the intestinal barrier
ginseng	⋁ *Bacteroidales, Verrucomicrobia* ⋀ Firmicutes
increased polysaccharides from vegetables	⋀ *Bacteroidetes* ⋁ *Firmicutes*
few fibers	⋀ *Enterobacteriaceae, Akkermansia muciniphila, Bacteroides caccae* ⋁ *Bacteroides ovatus, Eubacterium rectale*
fruits, vegetables, and fish, in case of overweight or obesity	⋀ Diversity

GUT MICROBIOTA AND AGING

Aging is a process leading to a generalized decline of physiological functions. This process depends on individual characteristics such as ethnicity, is genetically determined, and is modulated by environmental factors, including lifestyle, diet, medication use, and changes in gut microbiota.

With regards to microbiota variations, they are associated with enteric nervous system degeneration, intestinal motility alterations, and a reduction in the defense system against mucosal barrier dysfunction. There is an overall decrease in the microbiota's capability to ferment carbohydrates, whereas its capability to ferment proteins increases. Changes can involve both the **composition** and the **stability of gut microbiota.**

Normally, *Firmicutes* and *Bacteriodetes* are the most represented bacterial phyla in human gut microbiota, with *Firmicutes* as the predominant microorganisms in adults. However, in the elderly, the ratio is inverted; *Bacteriodetes* predominate, and the relative proportion of *Firmicutes* subgroups changes. Moreover, microbiota diversity decreases along with the abundance of species producing butyrate, which modulates the immune response by regulating inflammation mediators such as tumor necrosis factor α (TNF-α), interleukin IL-6, nitric oxide (NO) and IL-10. Also, among butyrate-producing reduced species is *Faecalibacterium prausnitzii,* a microbe protective against gut inflammation. *Bifidobacteria* decrease too, whereas levels of *Akkermansia muciniphila*, a mucin-degrading bacterium, increase. Finally, in centenarians it is possible to observe the enrichment in potential pathogens, particularly in *Proteobacteria*. All these changes are also associated with pro-inflammatory IL-6 and IL-8 blood concentrations.

Some gut microbiota changes, including the decrease of some butyrate-producing microbes and the increase of inflammation-associated species such as *Escherichia coli*, are less influenced by external factors than others; such changes may represent the core features of elderly gut microbiota and may be at least in part linked to the chronic activation of the immune system due to immunosenescence. Nevertheless, gut microbiota composition is also influenced by lifestyle factors such as diet.

First, a **less diverse diet** is linked to reduced gut microbiota diversity, and reduced diversity correlates with increased frailty, inflammatory markers, and impaired health parameters. Second, drastic dietary changes often occur with aging, resulting in the increase of sugars and fat-rich foods, and in reduced intake of plant-origin foods. A decreased consumption of healthy foods is associated with gut microbiota composition. An altered immune response to foods that promote inflammation may exacerbate microbial changes, and dietary factors altering the microbiome may exacerbate inflammation and alter immunity, leading to a two-way link between immunosenescence and dysbiosis.

Other factors that modulate gut microbiota in the elderly are **medications**, particularly antibiotics. These drugs influence gut microbiota along with the individual's residential location; beneficial microbes such as bifidobacteria and lactobacilli are more abundant, respectively, in community-dwelling and temporarily hospitalized individuals.

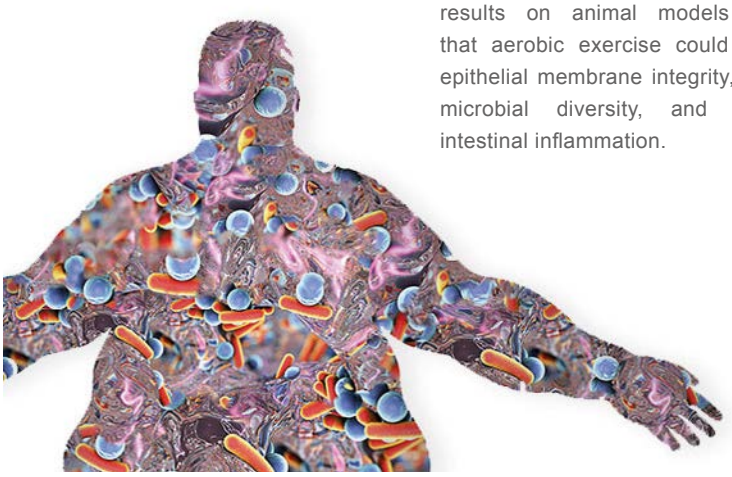
Finally, **intestinal permeability** can increase with age. Physical alterations of the epithelial barrier are associated with increased IL-6 concentrations and may play a role in dysbiosis and **inflammaging**, the low-grade pro-inflammatory state that is characteristic of aging associated with the expression of pro-inflammatory cytokines such as IL-6 and TNF-α.

During advanced age, the ability to resolve inflammation becomes impaired; this leads to the sustained presence of immunity cells (namely, leukocytes) in tissues, and to the chronic release of such pro-inflammatory molecules even in the absence of acute infection.

Gut microbiota is thought to have a role both in the induction and the maintenance of inflammaging, which has been hypothesized to increase the susceptibility to the development of different age-related diseases, including cancer. Its alterations can participate in this phenomenon in several ways. For example, the lack of *Akkermansia* and the increase of *Proteobacteria* is associated with the local and systemic inflammatory response, promoting small intestinal inflammation and systemic T cell activation. Conversely, it has been demonstrated that probiotics (live bacteria providing health benefits when consumed) exert beneficial effects on gut microflora composition and systemic immunity in the elderly.

Other microbiome-targeted interventions potentially leading to beneficial effects on age-related inflammation include caloric restriction, a Mediterranean-style diet implementation, and the use of nutritional supplements containing polyphenols such as resveratrol.

Besides diet, physical activity may be helpful, too. Few human studies have been published to date, but preliminary results on animal models suggest that aerobic exercise could enhance epithelial membrane integrity, increase microbial diversity, and attenuate intestinal inflammation.



MICROBIOTA MODIFYING FACTORS

Factors able to modify gut microbiota include different **medications**. Antibiotics are expected to have a large effect, but also other common medications can have an impact on gut microbiota composition.
Proton-pump inhibitors (PPIs) are, for example, associated with a higher *Streptococcaceae* and *Micrococcaceae* abundance and a lower microbiota diversity. Also, paracetamol and opioids are associated with an increase of *Streptococcaceae*, whereas serotonin reuptake inhibitors (SSRIs) are negatively associated with *Turicibacteraceae*. Moreover, inhaled anticholinergics are negatively associated with *Ruminococcaceae* and *Peptococcaceae* abundance and microbiota diversity.
Herbs can modulate gut microbiota too. For example, ginseng administration has been associated with the decrease in bacteria that possibly promote tumorigenesis (such as *Bacteroidales* and *Verrucomicrobia*) and an increase in bacteria that possibly exert anti-inflammatory and anti-cancer activities (such as *Firmicutes*).

Diet is another major regulator of gut microbiota structure and function. Its contribution to microbiota modulation and host-microbiota crosstalk is evident from the beginning of life, with human milk oligosaccharides participating in microbiota development and solid food introduction increasing bacterial richness. However, in the elderly, where food diversity is reduced, microbiota richness decreases, too. Some nutrients (such as glycans, quinones, and flavonoids) directly interact with bacteria, promoting or inhibiting their growth. Moreover, diet-derived compounds can indirectly shape gut microbiota by affecting host metabolism and the immune system. Vitamin D, for example, is associated with a decrease in circulatory levels of LPS, decreased abundance of *Coprococcus* and *Bifidobacterium*, and increased abundance of *Prevotella*, and dietary constituents (such as selected emulsifiers) might disrupt the intestinal barrier.
Carbohydrate restriction and diets rich in fiber and vegetables are associated with health benefits due, at least in part, to gut microbiota changes. High amounts of plant polysaccharides in the diet are associated with a low abundance of *Firmicutes* and a high abundance of *Bacteroidetes* (specifically *Prevotella*), whereas a paucity of dietary fiber is associated with the increase of *Enterobacteriaceae* (specifically S*higella* and *Escherichia*). The absence of dietary fiber is instead associated with an increase in mucus-degrading bacteria (*Akkermansia muciniphila* and *Bacteroides caccae*) at the expense of fiber-degrading species (*Bacteroides ovatus* and *Eubacterium rectale*).
That is why microbiota is responsive to some dietary interventions; for example, in overweight and obese people the consumption of fruits, vegetables, and fish is associated with microbiome richness. This is why the intake of prebiotics, probiotics, and symbiotic (the latter combining prebiotics with probiotics) has long been proposed as a way of modifying metabolic disorders largely dependent upon altered microbiota composition. Applying personally tailored diets is associated with shifts in gut microbiota composition after only 1 week of intervention.

Prebiotics are substances enhancing gut bacteria growth or activity. Among them are non-digestible dietary fibers (present in many fruits and vegetables) that are fermented by gut bacteria into SCFAs, and phytoestrogens (present, for example, in some berries). SCFAs, especially butyrate, help maintain intestinal immune homeostasis and protect from inflammation and carcinogenesis. Moreover, fiber seems to promote intestinal barrier function and improve glucose tolerance.
Prebiotic intake can significantly reduce body weight, body fat percentage, and desire for high-calorie foods. It may also improve insulin sensitivity, low-grade chronic inflammation, and lipid metabolism. Prebiotics can be administered in the form of fermentable dietary fiber such as inulin, oligofructose, fructooligosaccharides, or galactooligosaccharides, which can increase the abundance of bifidobacteria and lactobacilli, although not exclusively. Also, other substances like conjugated linoleic acid and milk sphingomyelin exert prebiotic activities.

Probiotics are instead live bacteria providing health benefits when consumed. They include lactobacilli and bifidobacteria and can be easily found both in fermented foods (such as yogurt and kefir) and in food supplements.
Dietary supplements with bacterial strains aim at replenishing the gut with healthy commensal bacteria granting favorable metabolic properties. They exert several and sometimes very different beneficial effects. In some cases, they can help reduce intestinal pain, bloating or tension, or attenuate the immune responses associated with acute colitis symptoms. Moreover, it seems that probiotics can help improve the intestinal barrier and inhibit pathogens growth. Given the central role played by gut microbiota in the brain-gut axis, probiotics can also help in cases of psychological diseases; specifically, they can reduce stress-associated visceral hypersensitivity, and lactobacilli and bifidobacteria can help fight anxiety and depression.
Strain mixtures might be more effective than some single-strain preparations.

THE BENEFITS OF PREBIOTICS

- Good immunity in the gut
- Protection of intestinal barrier integrity
- Enhanced glucose tolerance
- Enhanced insulin sensitivity
- Reduced body weight and fat mass
- Reduced craving for highly caloric food
- Reduced low-grade chronic inflammation
- Enhanced lipid metabolism

HOW TO ANALYSE GUT AND VAGINAL MICROBIOTA

Understanding the role of microbiota in maintaining health has been allowing for better disease prevention, diagnosis, and treatment. For example, healthiest gut microbiotas are resistant to invasion; conversely, gut microbiota vulnerability (that is the inverse of robustness) makes pathogenic invasion simpler. Thus, having a healthy, robust gut microbiota could help prevent pathogens from thriving. In other words, having a healthy, robust gut microbiota is desirable when antibiotic treatments are needed.

Specific microbiota compositions are associated with better health conditions. Unravelling individual profiles, **microbiota analysis** helps promote a good health status and prevent intestinal or systemic diseases (including cancer) acting on microbiota composition. Microbiota analysis can be useful to protect the health and well-being of and individual throughout life, from infancy to old age, and to develop individualized food plans and other lifestyle or, if needed, drug-based approaches aimed at correcting imbalances or improving microbiota composition.

First studies on microbes – including those living in the gut – predominantly focused on individual species cultured in the laboratory. The first sequenced microbial genome (that of *Haemophilus influenzae*) was published in 1995. However, the vast majority of microbes – including bacteria living in the gut – cannot be cultured in the laboratory, and therefore cannot be studied with classical microbiological methods. Moreover, culturing favors the selection of microbes best able to thrive under laboratory conditions, and not necessarily the dominant or the most influential one in the gut. Moreover, in nature, many microbes function as multicellular – and, often, multispecies – entities, interacting and communicating in complex ways.

Today, we are in a new era, called **metagenomics**, in which the power of genomics (the study of the entire genetic material of an organism), bioinformatics, and system biology are combined to analyze the entire community of gut microbiota, bypassing individual microbial isolation and culture. Metagenomics transcends the individual microbe, focusing on genes and their reciprocal influence in the gut microbiota community. New tools enable studying gut microbes in the complex community where they actually live, analyzing the genome of many microorganisms simultaneously. This allows understanding what gut microbiota are capable of, how they work, and the alterations that could lead to health problems.

The first truly metagenomic survey of human gut microbiota appeared in 2006, and the first catalog of human microbiota bacterial genome, comprising 178 references, was published in 2010 by the Human Microbiome Project; until 2017, 437 gut microbiota genomes were sequenced. However, more than half of the sequences obtained from the analysis of a human gut microbiome cannot be mapped to existing bacterial reference genomes.

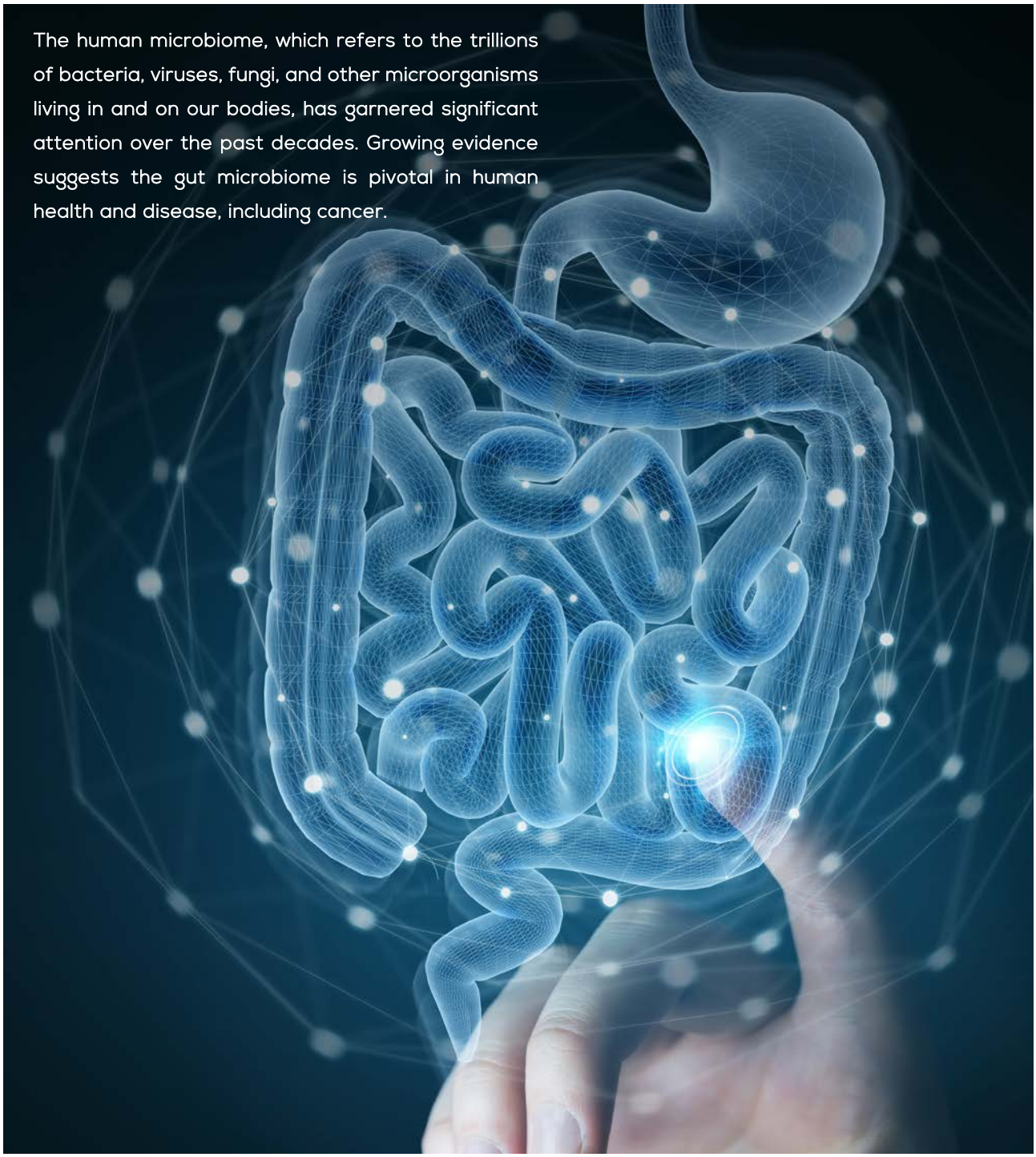
In 2019 a reference catalog of 1,520 nonredundant, high-quality draft bacterial genomes of human gut bacteria isolated using different culturing conditions (the Culturable Genome Reference) deposited in the China National GeneBank (CNGB) improved the mapping rate of selected metagenomics datasets to over 70%.

The first step of a gut microbiota metagenomics study is DNA extraction from stool samples. The following DNA sequencing can capture a massive amount of information on gut microbiome. However, to study microbiota composition and diversity it is possible to focus on so-called ribosomal RNA (rRNA) phylotyping, a culture-independent method based on a database of more than 200,000 rRNA gene sequences.

rRNAs are essential components of cellular protein-making engines, ribosomes. All organisms, including bacteria, have rRNAs that are different enough to be distinguished one from another. Thus, the analysis of rRNA sequences in a stool sample allows for identifying gut microbiota composition. Focusing only on one gene, rRNA phylotyping represents a useful preliminary step to providing an assessment of gut microbiota diversity.

MICROBALANCE

In this scenario Bioscience Institute presents MICROBALANCE, a test based on the analysis of 16S rRNA gene, the DNA sequence encoding the smaller ribosome subunit of bacterial rRNA.
Individuals are provided with a kit for stool or vaginal fluid sampling and transport which is then sent to Bioscience's laboratories for bacterial DNA extraction and 16S rRNA gene library preparation.
The 16S rRNA gene is sequenced in Bioscience's laboratories by Next Generation Sequencing with ION S5™ System (Thermo Fisher Scientific). Obtained sequences are subjected to bioinformatic analysis.
Sequencing output does not represent the diagnosis of a pathology, but a microbiota profile allowing medical doctors and qualified nutritionists to identify food plans and lifestyle adjustments aimed at correcting imbalances or improving microbiota composition.



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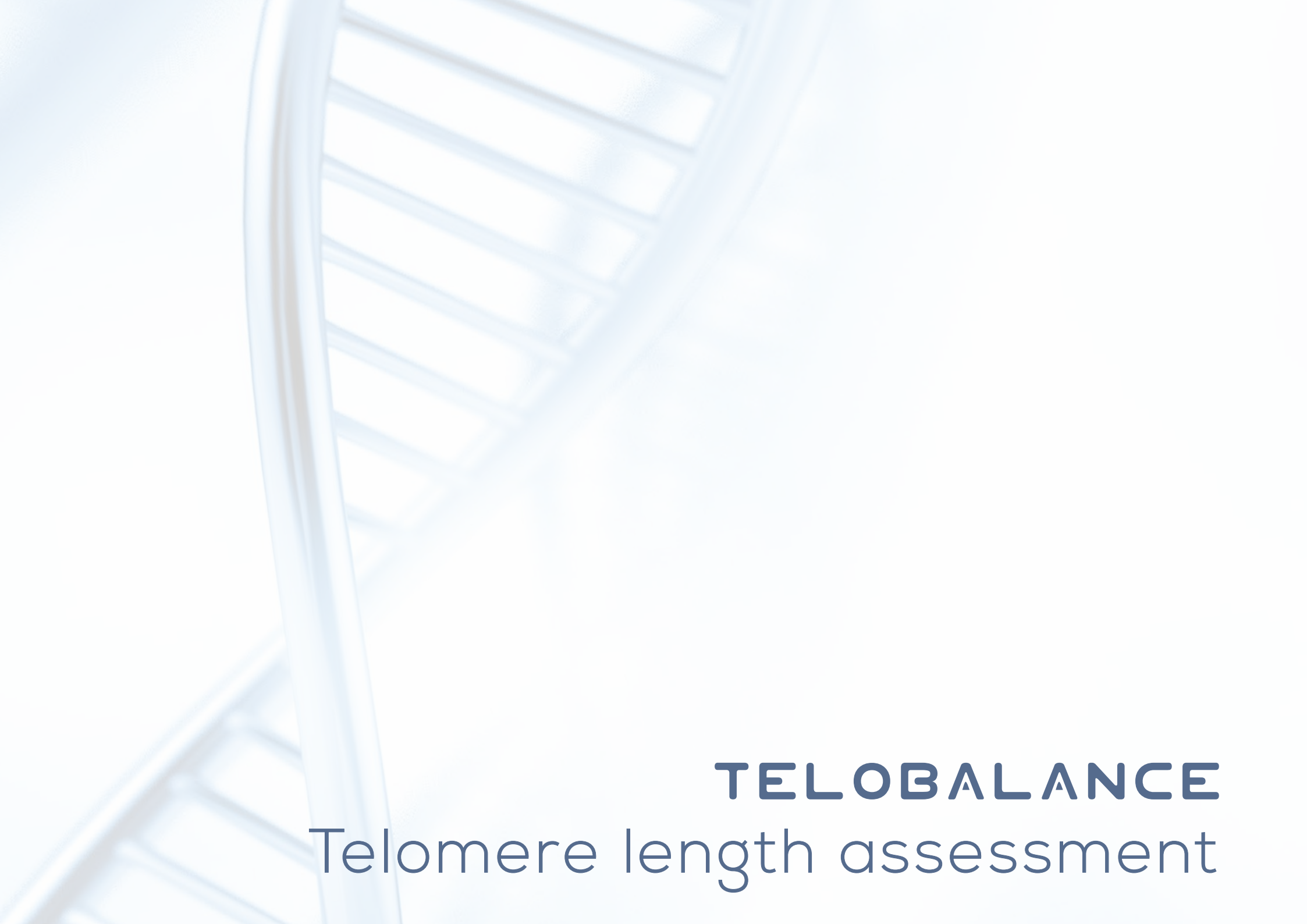

TELOBALANCE
Telomere length assessment

TELOBALANCE

INTRODUCTION

Deoxyribonucleic acid (DNA) is the code of life. For each human cell, its total length exceeds 2 meters, but its peculiar organization allows it to be stored in a nucleus of just 10 micrometers in diameter. In fact, human DNA is condensed with proteins into compacted units called chromosomes, clearly visible with a microscope during cell division. This high degree of condensation allows the human body to contain a quantity of DNA that, if unwound, would be sufficient to cover more than 41 times the Earth-Sun distance.

Telomeres are the repetitive DNA-protein complexes that protect the ends of chromosomes. These specialized capping structures avoid both the recognition of chromosome ends as sites of DNA damage and chromosome end fusion. Their function depends on a minimal length of DNA repeats and on the functionality of the associated protein complexes.

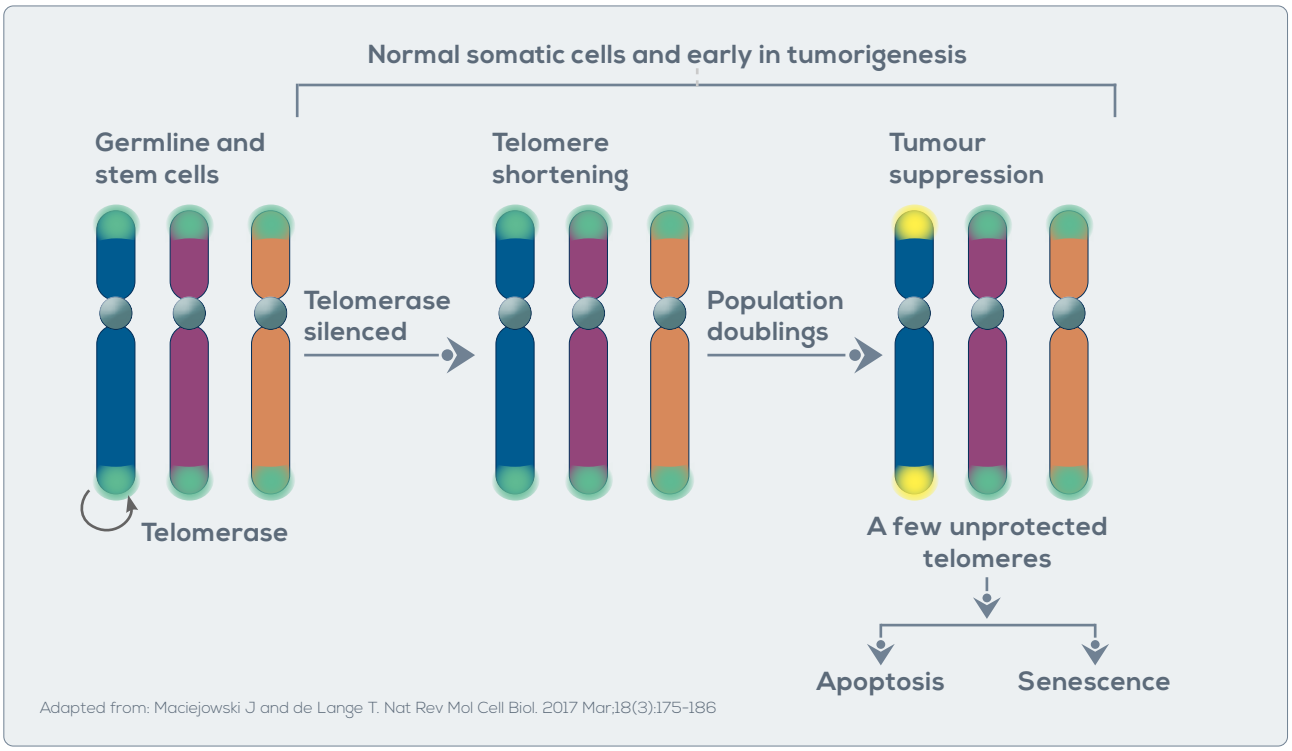
Unfortunately, during progressive cell divisions telomeres critically shorten. This phenomenon generates dysfunctional structures able to elicit DNA damage responses. Consequently, cellular senescence is triggered, and cells undergo an essentially irreversible growth arrest.

Cells are equipped with the machinery required to avoid telomere attrition. In somatic cells this machinery is turned off to allow growth arrest in the presence of critically shortened telomeres. However, when cells gain oncogenic changes, they can bypass the senescence triggered by dysfunctional telomeres. They continue to divide, entering a period of an extended lifespan.

In particular, the risk of genetically unstable cell proliferation is increased in cells that accumulate mutations impeding growth arrest and cell death induction. In such a situation, critically shortened telomeres can promote genomic instability, the principal driver of cancer development.

TELOMERE STRUCTURE AND FUNCTIONS

Telomeres represent both genomic sacrifice zones, which prevent the loss of genomic information at the end of chromosomes, and high order structures masking chromosome ends from being detected as a site of damaged DNA by the DNA damage response machinery. Also, telomeres regulate gene expression, transcriptionally silencing genes both close to them and/or at long distances.
These structures are characterized by the presence of a terminal single-stranded DNA overhang which folds back invading the preceding repetitive homologous double-stranded region, forming a telomeric loop (T-loop). This conformation protects the otherwise exposed chromosome end from being recognized as a site of DNA cleavage. Also, the proteins complexed with DNA (TRF1, TRF2, RAP1, TIN2, TPP1, and POT1, collectively named the shelterin complex) binds the repetitive double-stranded sequences and the single-stranded overhang and allows the DNA damage repair machinery to distinguish telomeres from sites of DNA breakage.
Other higher-order DNA conformations, such as four-stranded structures (the so-called G-quadruplexes), and a long non-coding RNA transcribed from telomere DNA (the telomere repeat-containing RNA) are thought to be implicated in normal telomere function and maintenance.
Human telomere length is heterogenous. Cell viability and chromosome stability depend on the shortest telomere.



Adapted from: Maciejowski J and de Lange T. Nat Rev Mol Cell Biol. 2017 Mar;18(3):175-186

TELOMERES IN CANCER

Human telomere length encompasses a normal range with discrete upper and lower limits. Both longer and shorter telomeres have been linked to cancer.
In the first case, the association lies in the presence of mutations promoting telomere elongation. Longer telomeres may favor a delayed cell senescence, increasing the opportunities to develop genomic instability, thus the risk of carcinogenic transformation.
On the other hand, shorter telomeres can be a direct source of genomic instability. Unstable, dysfunctional telomeres are recognized as DNA damage sites and can promote chromosomal rearrangements ultimately leading to genome alterations that favor cancer development.
In both cases, telomere length plays a pivotal role in the survival of cancer cells.

TELOMERE SHORTENING AND CANCER

Telomere shortening is a natural phenomenon that occurs with aging. Its cause lies in the mechanism of DNA replication. In fact, one of the two strands of the DNA double helix cannot be copied all the way to its very end, leading to the loss of part of the protective cap of the chromosome at every cell cycle (50-200 bp, depending on the tissue). After 50 to 60 cell divisions, telomeres are short enough to uncap the chromosome end, and their shortening can promote genome instability.

Inflammation and **oxidative stress**, two major pathways of carcinogenesis, accelerate telomere shortening. Moreover, the progressive reduction of telomere length is particularly dangerous when combined with changes such as loss of the function of p53, a protein that inhibits cell proliferation and induces senescence, or of other cell-cycle regulators.

In fact, during the period of replicative senescence (or mortality stage 1, M1) that follows the uncapping of one or two shortened telomeres, cellular proliferation is usually inhibited. However, oncogenic changes can promote an M1 bypass, thus extending the cell division period during which telomeres can further shorten.

During this new dysfunctional state (the M2 crisis), chromosome end-to-end fusions can occur. The cell death triggered by this situation protects from cancer development. However, between 1 in 100,000 and 1 in 10 million cells can develop mechanisms to maintain its very short telomeres, bypassing the M2 crisis and becoming immortalized.

Moreover, maintaining shortened telomeres allows the cell to express specific genes required for cancer progression that would be otherwise silenced by the conformation of telomeres.

Currently, these complex mechanisms are considered as both biomarkers and potential targets for cancer diagnosis and treatment. In 85-95% of cancers, they involve the activation of the ribonucleoprotein enzyme telomerase.

Usually inactive in the vast majority of somatic cells, telomerase is able to synthesize the typical DNA repeats located at the end of chromosomes. It works as a reverse transcriptase – that is, it synthesizes DNA based on an RNA template. This RNA template is an essential component of telomerase known as human telomerase RNA (**hTR**) or human telomerase RNA component (**hTERC**).

Besides being the template for telomerase action, hTERC is involved in the catalysis, the localization, and the assembly of the enzyme too. The other essential component is the catalytic protein subunit, known as telomerase reverse transcriptase (TERT) and encoded by the ***hTERT*** gene. In the holoenzyme, TERT and hTERC interact with accessory proteins such as DKC1, NOP10, NHP2, pontin/reptin, and GAR1.

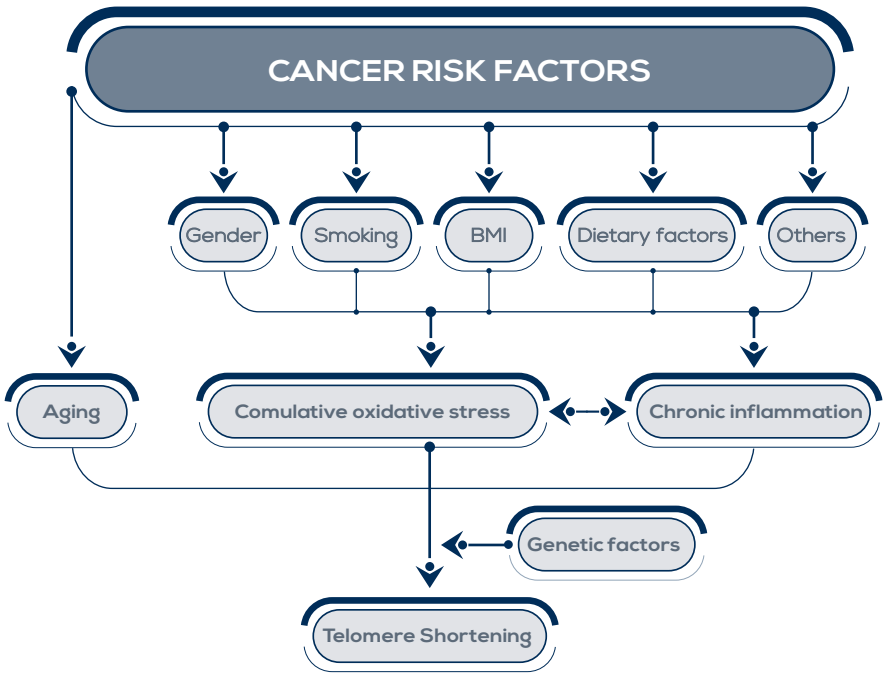
hTERC is constitutively expressed in somatic cells, whereas *hTERT* is epigenetically silenced, representing a limiting factor. The consequent repression of telomerase activity is a tumor suppression pathway that limits cancer cell development.

Evidence in support of this tumor suppression activity includes the association between longer telomere length and increased B cell lymphoma and chronic lymphocytic leukemia risk, as well as the association between genetic determinants of long telomeres and increased overall risk of cancer (especially melanoma and lung cancer).

Acquisition of telomerase activity by cancer cells is achieved by re-expression of *hTERT*. Promoter mutations are present in multiple cancer types (e.g. melanoma, pleomorphic dermal sarcoma, myxoid liposarcoma, glioma, urothelial cell carcinoma, carcinoma of the skin, liver cancer, gastric cancer, pancreatic cancer, non-small cell lung cancer, gastrointestinal stromal tumors) and have been detected across all stages and grades of the disease, suggesting they are an early event in the process of cancer development. However, they are not indispensable: in cancer cells without these mutations, telomerase activity maintains the telomeres, too. In some cases, telomerase activity is further enhanced by other gene mutations.

Beside promoter mutations, other mechanisms control telomerase expression (e.g. genomic rearrangements, alternative splicing, DNA methylation, histone modification).

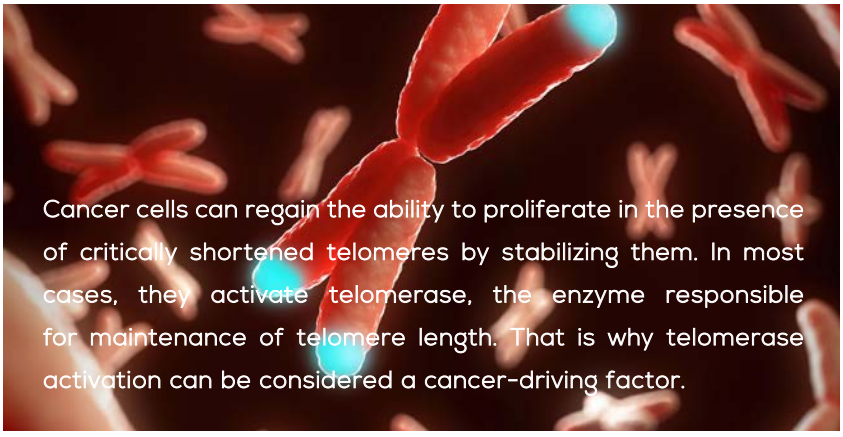
Adapted from: L. Hou et al. Cancer Letters 319 (2012) 130-135



Finally, in some cell types *hTERC* expression is a limiting factor, and the expression of both *hTERT* and *hTERC* is needed for a robust telomerase activity.

The telomere elongating ability of cancer cells does not necessarily correspond to longer telomeres. Indeed, in a 2017 study that analyzed telomere length across 31 types of cancer, shorter telomeres were present in 70% of them.
In fact, telomerase preferentially elongates the shortest telomeres. Available data suggest that immediately after the acquisition of *hTERT* promoter mutations, telomerase activation is not sufficient to elongate all telomeres. Thus, the enzyme activity concentrates on the shortest telomeres, which are elongated; meanwhile, longer telomeres shorten, resulting in gradual attrition of all the telomeres in the cell. After telomeres have shortened, the activity of the enzyme would increase to levels that are sufficient to stabilize their length.
It is also possible that during the development of cancer various cell populations arise, with long to short telomeres, and that only the cells with shortest telomeres increase telomerase activity to maintain them and avoid crisis. Furthermore, shelterin proteins may play a role by stabilizing shorter telomeres. Compared to the non-cancerous cells, many cancer cells are characterized by elevated shelterin levels, and their expression correlates with the progression level of the tumor.

Unfortunately, cancers with shorter telomeres are characterized by a poor prognosis. Moreover, alteration of telomere length seems to affect the morphology of cancer tissue and the expression of cancer-associated genes. Thus, besides avoiding the M2 crisis, shortened telomeres might contribute to the severity of the tumor by influencing its genetic signature.
Telomere length maintenance is not the only way telomerase participates in cancer development. In fact, this enzyme is involved in other activities that are thought to significantly contribute to oncogenesis (gene expression regulation, cell proliferation, apoptosis, WNT/β-catenin signaling, NF-kB signaling, MYC-driven oncogenesis, DNA damage repair, cell adhesion and migration, epithelial–mesenchymal transition).
Because of its expression in the majority of cancer types and in cancer stem or stem-like cells, and because of its low activity in normal cells (including stem cells), anti-telomerase therapeutics could selectively induce cell death in cancer cells, sparing or minimizing the effect on non-cancerous cells. Molecules such as competitive inhibitors of telomerase or immunotherapeutic drugs were developed for the treatment of various cancers. Also, telomerase-mediated telomere-disrupting approaches, such as the use of modified nucleosides, which are incorporated into telomeric DNA by telomerase leading to telomere dysfunction, may provide a valuable option for cancer treatment.
It should be noted that a limited number of cancer cells (5-15%) develop mechanisms other than telomerase activation to maintain shortened telomeres. These mechanisms are referred to as **Alternative Lengthening of Telomeres (ALT)** and involve the use of DNA recombination to extend telomeres length.
For example, 20 to 65% of sarcomas elongate telomeres by activating ALT.



CANCERS MOST FREQUENTLY ASSOCIATED WITH *hTERT* PROMOTER MUTATIONS

- bladder carcinoma
- renal pelvic carcinoma
- urothelial carcinoma
- hepatocellular carcinoma
- melanoma
- skin basal cell carcinoma
- thyroid cancer (papillary and poorly differentiated carcinomas)
- myxoid liposarcoma
- glioblastoma
- medulloblastoma
- oligoastrocytoma
- oligodendroglioma

TELOMERE SHORTENING AND GENOMIC INSTABILITY

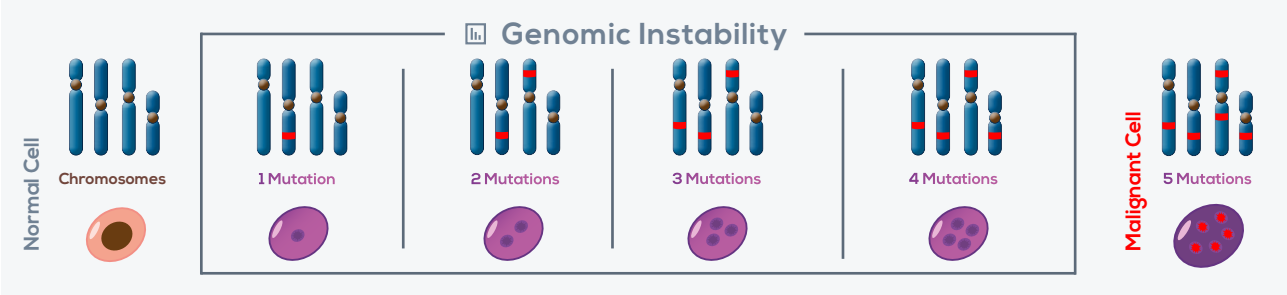
Cancer is a genetic disease that manifests when cells accumulate **genomic instability** – the principal driver of cancer development – over a period of time and acquire replicative immortality. In combination with other oncogenic changes, telomere shortening can promote chromosomal instability, significantly contributing to genomic rearrangements associated with tumorigenesis.

In fact, by protecting chromosome ends, telomeres avoid end-to-end fusion during a normal cell's life. However, during the M2 crisis, breakage-fusion-bridge cycles take place. Sister chromatids lacking protective ends originated by DNA replication fuse together. This phenomenon gives rise to the formation of a bridge of DNA that interferes with the correct segregation of sister chromatids during cell division. When the bridge breaks, uneven derivative chromosomes form, and in the daughter cell a second fusion occurs, developing further genomic instability.

In the majority of cells, high genomic instability leads to cell death. However, in rare cells the activation of telomerase enables escaping from telomere crises. Breakage-fusion-bridge cycles are interrupted by telomerase-mediated chromosome end healing. This leads to the generation of transformed cells with a heavily rearranged, stabilized genome enriched with potentially tumorigenic mutations.

Available data link telomere dysfunction to nearly all cancer-related genome alterations: gain and loss of chromosome (aneuploidy), translocations, gene loss, regional amplifications, whole genome reduplication (tetraploidy), chromothripsis (tens to hundreds of genomic rearrangements in one or few segments of DNA), and kataegis (a specific hypermutated pattern often accompanying chromothripsis).

To produce many of these rearrangements and to wreak havoc on the genome, it is sufficient to lose the telomere at individual chromosome ends.



LONGER TELOMERES AND CANCER

Cancer is only a rare (10% to 15%) complication of the so-called **short telomere syndromes,** which consist of a series of clinical presentations which can manifest from infancy (immunodeficiencies, bone marrow failure, enteropathies, idiopathic pulmonary fibrosis, emphysema, and liver diseases) associated with telomerase and telomere maintenance genes mutations. Probably, in the case of degenerative diseases, and not cancer, they are the predominant consequences of telomere shortening because in most patients with short telomere syndromes the DNA damage response is intact.

Other mutations influencing telomere elongation are associated with the so-called **long telomere syndromes**, which are associated with **familial cancer**. Longer telomeres resulting from these mutations confer a longevity advantage and may permit an increased replicative potential of cells acquiring mutations that would otherwise undergo cell death, thus, promoting cancer development. The most prevalent cancers in the carriers of these *hTERT* and shelterin genes mutations are melanoma and chronic lymphocytic leukemia. However, even if such mutations were identified in familial forms of a specific cancer, people carrying it showed other malignancies, too. This suggests that this kind of alteration confers a broader cancer-prone state.

Furthermore, other mechanisms have been suggested associating longer telomeres with cancer. In the case of breast cancer, a hormone-related disease, reports of longer telomeres in women than men, and in postmenopausal women with hormone replacement therapy suggest that estrogen might play a role in determining telomere length in breast cancer. Moreover, telomere length maintenance might be promoted by the antioxidant capacity of estrogen.

Finally, it has been suggested that in renal cell carcinoma the downregulation of the immune response might reduce telomere attrition, leading to longer telomeres.

TELOBALANCE

Examples of monitoring telomere length in blood cell DNA as a surrogate biomarker of cancer exist in the literature. Even more so, telomere length in circulating cell-free DNA (cfDNA) was associated with cancer risk.

However, the measurement of bulk telomere length is an imprecise predictor of cell cancer potential. In fact, human telomeres are heterogeneously sized, and in a cell with an apparently ample telomere reserve, there can be several very short telomeres.

Evaluating the percentage of short telomeres and the distribution of telomere lengths is a more useful approach.

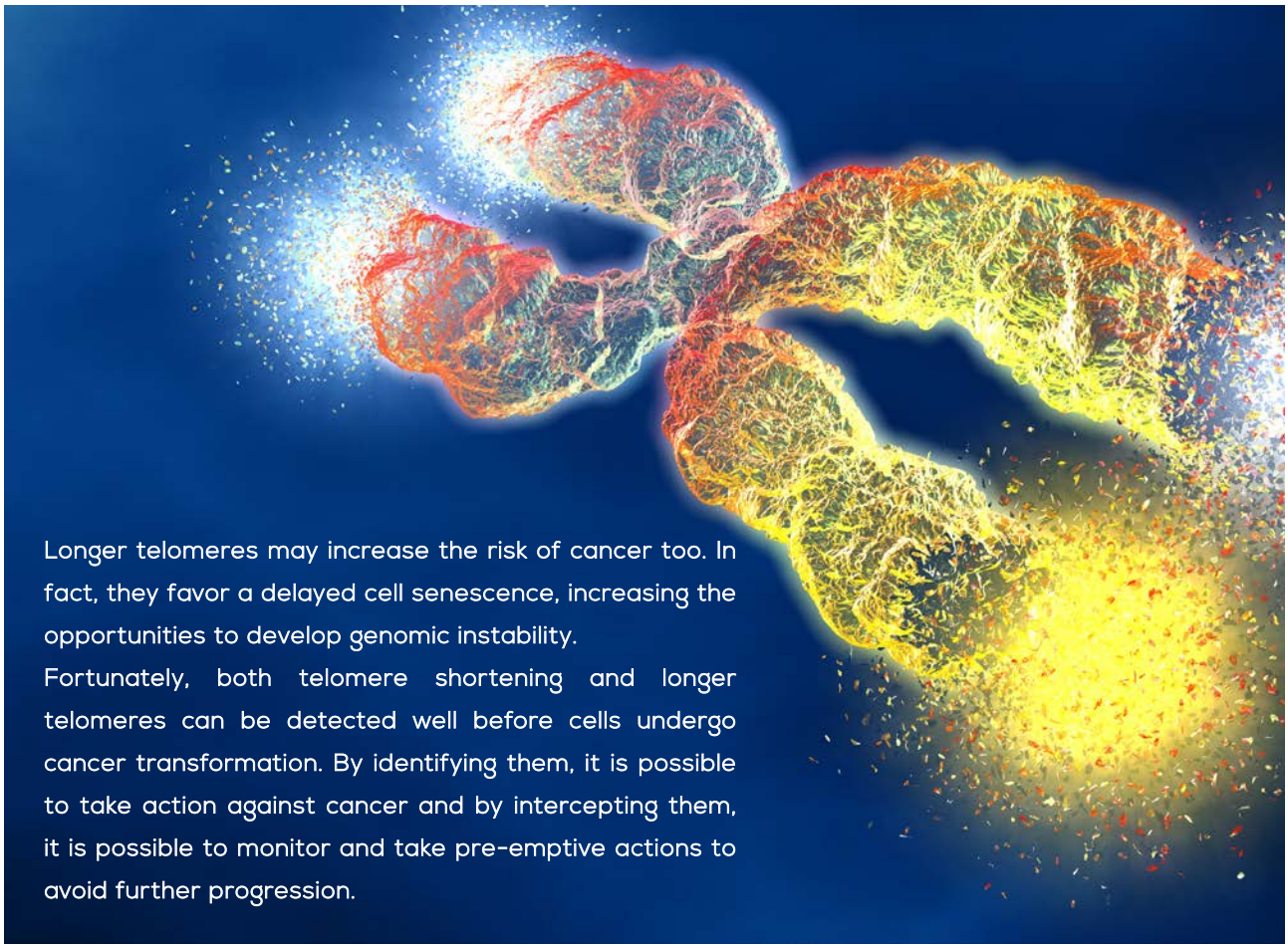
By measuring telomere length chromosome by chromosome, and by evaluating about 200 telomere length associated variables, **TELOBALANCE** provides a picture of the median and mean telomere length in the sample, the percentage of short telomeres, the percentage of cells with shortened telomeres, and other parameters that are useful in determining the level of telomere attrition in the cells. The assay is based upon a robust and reproducible high-throughput quantitative fluorescence *in situ* hybridization technology. Telomere length is measured for individual chromosomes, with each sample analyzed in multiple replicates to achieve statistical significance.

Moreover, relative telomerase activity is measured by the Telomeric Repeat Amplification Protocol (TRAP), modified for real-time, quantitative, PCR (qPCR) analysis (Q-TRAP). Telomerase is extracted and incubated with its specific substrate. The products of the reaction are then quantified by real-time qPCR. The assay is routinely performed in triplicate, ensuring that the data are both reproducible and quantitative.

Finally, it is possible to measure *hTERT* expression too. The assay is based on a custom TaqMan gene expression real-time PCR. To ensure that the data are both reproducible and quantitative, the test is routinely performed in triplicate.

TELOBALANCE results do not represent a diagnosis, but they allow intercepting conditions that can contribute to cancer development, anticipating it and enabling action to counteract it.

Given the association between telomere dysfunction not only with aging but also with smoking, dietary factors, body mass index, and other factors promoting oxidative stress and chronic inflammation, it is recommended to people of every age, to actively monitor cancer development based upon the interception of its driving factors.

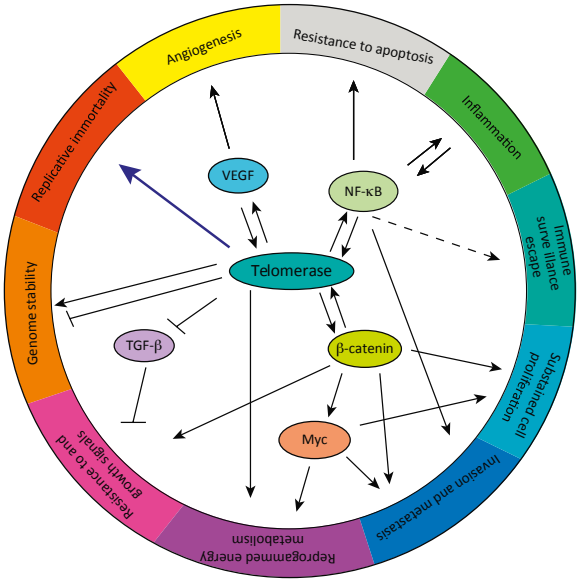


Longer telomeres may increase the risk of cancer too. In fact, they favor a delayed cell senescence, increasing the opportunities to develop genomic instability.

Fortunately, both telomere shortening and longer telomeres can be detected well before cells undergo cancer transformation. By identifying them, it is possible to take action against cancer and by intercepting them, it is possible to monitor and take pre-emptive actions to avoid further progression.

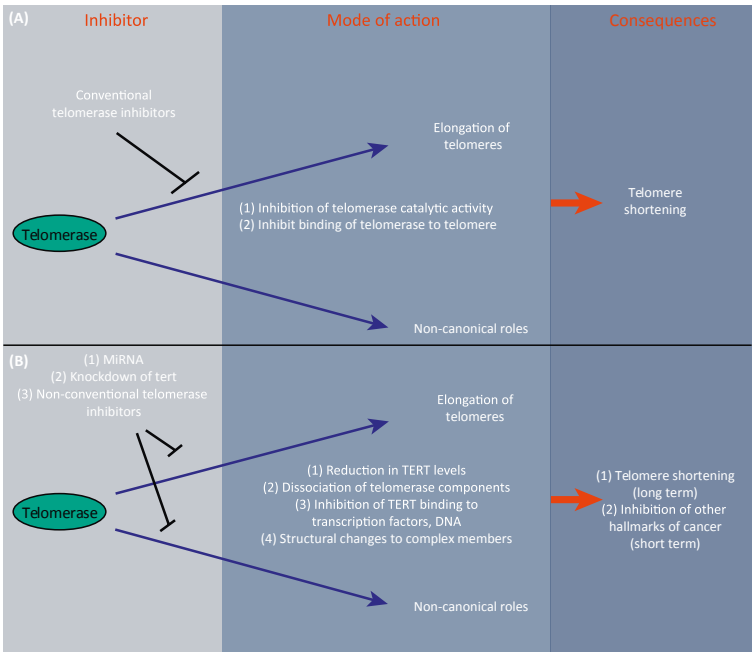
CENTRAL REGULATOR OF ALL OF THE HALLMARKS OF CANCER

Regulation of the hallmarks of cancer by telomerase. TERT regulates the various hallmarks of cancer through its enhancement of transcription of VEGF, NF-kB and b-catenin target genes. b-Catenin also activates Myc. TERT also suppresses TGF-b-dependent growth suppression. Telomerase also antagonizes or suppresses genome instability, in contrast to the other effects, and that this might be through a transcription-independent mechanism (please see text for details). Arrows not drawn to scale. NF-kB, nuclear factor k-light-chain-enhancer of activated B cells; TGF-b, transforming growth factor-b; VEGF, vascular endothelial growth factor. (⟶ : Direct activation ⇢ : Indirect activation. ⟞ : Inhibition. ⇄ : Two-way activation. ⟶ : Canonical telomerase activity.)



Adapted from:Telomerase: central regulator of all of the hallmarks of cancer; K Low and V Tergaonkar.

Therapeutics against telomerase. (A) Conventional telomerase inhibitors inhibit the canonical roles of telomerase by negating its catalytic activity or inhibiting its binding to telomere ends, leading to shortening of telomeres, but do not inhibit its noncanonical roles. (B) Cell proliferation, metastasis, and telomere lengthening might be better controlled through the use of miRNAs that downregulate TERT concurrently with other oncogenes, nonconventional telomerase inhibitors that affect its noncanonical roles, and/or drugs that knockdown telomerase levels. These inhibitors downregulate TERT levels, dissociate telomerase complex or inhibit its interaction with transcription factors.



Adapted from:Telomerase: central regulator of all of the hallmarks of cancer; K Low and V Tergaonkar.

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CHEMOPREVENTION
Chemopreventive Agents

CHEMOPREVENTION

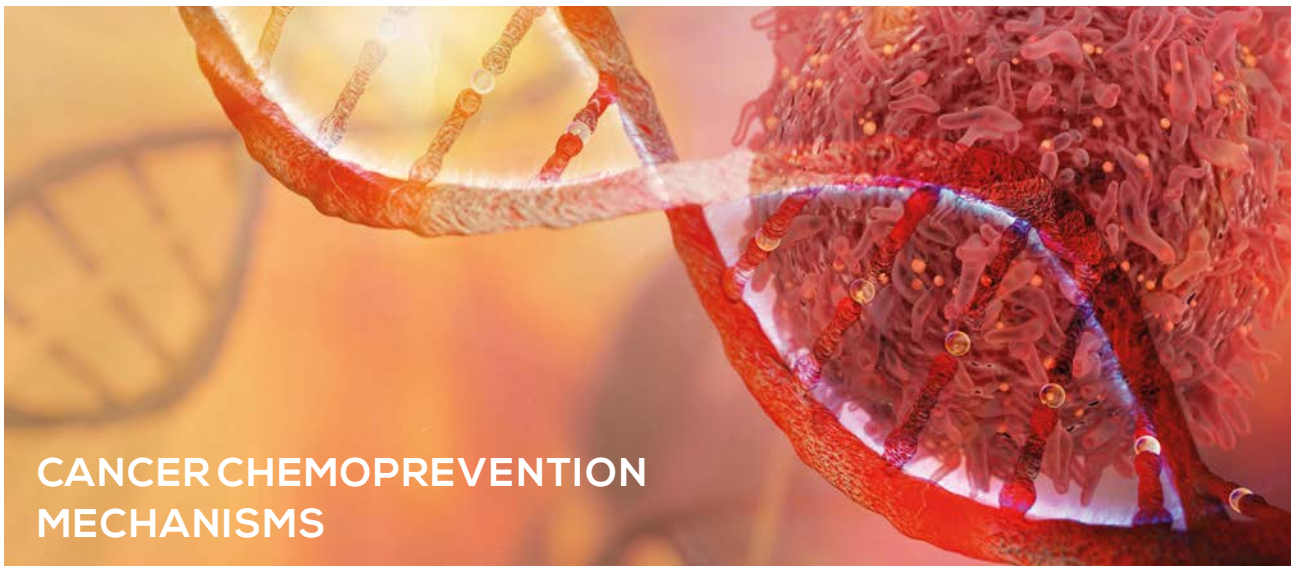
INTRODUCTION

Cancer prevention is based on a three level-approach. Both secondary and tertiary preventions allow for acting on an already formed cancer mass, when it is, respectively, in its early or advanced stages. But only primary prevention enables counteracting cancer development, representing the easiest and most effective way of fighting it. In fact, primary prevention is aimed at eliminating or mitigating cancer risk factors. Some approaches are somewhat passive, such as avoiding alcohol and tobacco use, whereas others are more active, such as exercising, eating a healthy, balanced diet, applying UV-sunscreens, and chemoprevention.

Chemoprevention involves the implementation into the body of external agents, such as drugs and dietary supplements, to prevent or delay tumor onset. It moves the therapeutic target down to the spectrum of carcinogenesis, allowing for achieving high levels of cancer control. This approach is particularly suited for high-risk individuals, such as people that are discovered to be carriers of genome instability or of other conditions (such as low-grade chronic inflammation, immune imbalances, dysbiosis) that are associated with an increased cancer risk.

The use of chemopreventive agents is based upon a fundamental principle of cancer biology: as a cell progresses through carcinogenesis, genome instability and resultant functional aberrations exponentially increase. This means that during carcinogenesis the number of dysregulated processes needing to be reverted to avoid cancer development exponentially increases too.

Another tenet of chemoprevention depends on a basic principle of pharmacology: any therapeutic agent acts on a limited spectrum of targets (usually one). That means that, as cancer development progresses, more than one therapeutic agent, acting on different functional aberrations, is usually needed to counteract the disease.



CANCER CHEMOPREVENTION MECHANISMS

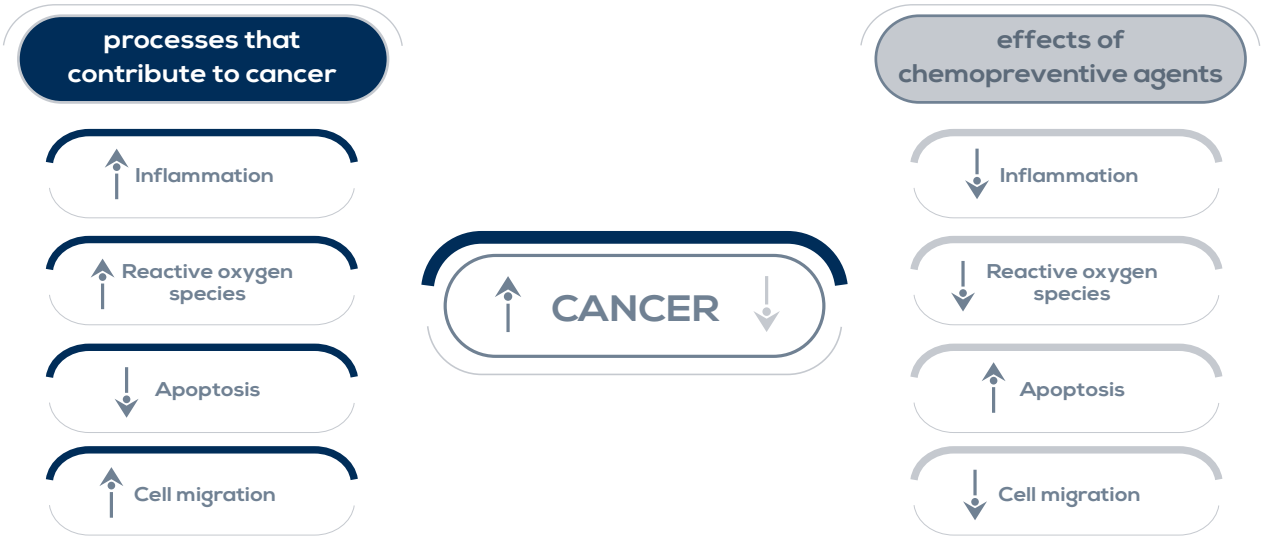
Chemopreventive agents include a wide range of compounds of both natural and synthetic origin. Often used for purposes other than cancer treatment, their chemopreventive potential against cancer emerged from observation of dietary habits of populations presenting low incidence of specific tumors, epidemiological or clinical studies of drugs showing decreased incidence of cancer as a secondary effect, or laboratory screening on tumor cell cultures showing the induction of surrogate markers of a malignant-to-normal reversion.

These chemopreventive compounds can act in several ways. They can block cancer-causing agents from modifying DNA, promote the activity of the DNA repair system, or act on cells that carry cancer-associated DNA mutations or characterized by genome instability. Their classical mechanisms of action include the reduction of **oxidative stress**, the control of **chronic inflammation**, and the downregulation of specific **cancer-associated pathways.**

Oxidative stress can damage DNA, promoting cancer-associated mutations and genome instability. Chemopreventive agents can reduce it by activating free radical scavenging enzymes or by directly acting as a free radical scavenger.

By counteracting inflammation, chemopreventive agents counteract DNA damage too. In fact, inflammation can trigger oxidative stress, thus DNA damage. Moreover, inflammatory molecules such as NF-kB influence the activity of genes involved in cancer development.

Finally, almost all cancer cells harbor epigenetic modification – that is, alterations that influence gene expression via DNA remodeling, without any change in gene sequence. Several chemopreventive compounds – most of natural origin, such as resveratrol, sulphoraphane, isoflavones, and green tea-derived molecules – seem to counteract cancer development by epigenetics, influencing cancer cell proliferation, survival, and death.



Effects of chemopreventive agents on processes that contribute to tumorigenesis and cancer progression.

Adapted from: Maciejowski J and de Lange T. Nat Rev Mol Cell Biol. 2017 Mar;18(3):175-186

EXAMPLES OF DRUGS ALREADY USED IN CANCER CHEMOPREVENTION

Evidence for cancer incidence reduction in high-risk populations exists for several chemopreventive agents, such as selective estrogen receptor modulators (SERMs, tamoxifen and raloxifene) against breast cancer, non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and celecoxib) against colorectal cancer, and vaccines (anti-hepatitis B virus – HBV – and anti-papillomavirus – HPV) against virally induced cancers (respectively, hepatocellular carcinoma and cervical, oropharyngeal, anal, penile, vulvar, and vaginal cancers).

The first FDA-approved chemopreventive agent is tamoxifen. In phase III cancer prevention trials, tamoxifen treated high-risk pre- and post-menopausal women showed a 30-60% reduction of estrogen receptor (ER)-positive breast cancer. Raloxifene showed a similar chemopreventive effect. Both observational studies and randomized controlled clinical trials associated aspirin use with decreased incidence and mortality of colorectal cancer in the general population. A smaller effect was observed also on the incidence of stomach and esophageal cancers. Finally, HBV and HPV vaccines are currently the most successful implementations of cancer preventive vaccines. These and other examples of FDA-approved chemopreventive drugs are listed in Table 1.

Table 1. Examples of FDA - approved chemopreventive agents and interventions that target cancer development associated viruses and bacteria.

Adapted from: Al Rabadi L and Bergan R. Cancer Prev Res (Phila). 2017 Jan;10(1):14-35

DRUG	CANCER	MECHANISM
Tamoxifen	Breast	Selective estrogen receptor modulation
Raloxifene	Breast	Selective estrogen receptor modulation
Porfimer sodium + photodynamic therapy & omeprazole	Esophageal	Production of oxygen free radicals
Aspirin	Colorectal	Apoptosis induction and prostaglandin production interruption
Celecoxib	Colorectal	Apoptosis induction and prostaglandin production interruption
Bacillus Calmette-Guerin	Bladder	Induction of local immune reaction against the tumor
Valrubicin	Bladder	DNA synthesis inhibition and cell death
Fluorouracil	Skin	DNA synthesis inhibition and cell death
Diclofenac sodium 3%	Skin	Unknown
5-aminolevulinic acid + photodynamic therapy	Skin	Precancerous cell killing
Imiquimod	Skin	Immune response enhancement and apoptosis promotion
Ingenol mebutate	Skin	Primary necrosis induction
Gardasil 9 vaccine - HPV - 6, 11, 16, 18, 31, 33, 45, 52, 58 Cervarix vaccine - HPV - 16, 18	Cervix , Vulva, Vagina, Anus (only Gardasil 9 approved in 9-15 years old males)	Immune reaction against human papillomavirus (HPV)
Hepatitis B vaccine	Hepatocellular carcinoma	Immune reaction against hepatitis B virus
Interferon therapy Nucleoside analogues		Hepatitis B virus life cycle blockage
Interferon therapy Nucleoside analogues	Hepatocellular carcinoma	Hepatitis C virus life cycle blockage
Antiretroviral therapy	Non-Hodgkin, lymphoma Kaposi Sarcoma	Human immunodeficiency virus and human herpesvirus 8 life cycle blockage
Antibiotics	Stomach	Counteracting *Helicobacter pylori*
Antischistosomal	Bladder	Counteracting parasitic worms responsible for schistosomiasis
Antihelminthic	Cholangiocarcinoma	Counteracting *Clonorchis sinensis* and *Opisthorchis viverrini*

Other drugs, not yet officially approved to lower cancer risk, showed chemopreventive potential against tumors. For example, randomized clinical trials on high-risk post-menopausal women showed that **aromatase inhibitors** (AIs, anastrozole and exemestane) produce a stronger efficacy than SERMs against ER-positive breast cancer. **Statins**, the gold standard treatment for coronary heart disease prevention, could decrease the long-term incidence of colorectal and gastric cancer. And **metformin**, the common drug for type II diabetes, was associated with a 31% reduction in overall cancer incidence and a 34% reduction in cancer mortality. Among other organs that metformin would protect from cancer are breast, colon, liver, pancreas, prostate, endometrium, and lung.

THE IDEAL CHEMOPREVENTIVE TREATMENT

As it has to be used on healthy people who did not yet develop the diseases, the ideal chemopreventive agent has little or no toxicity. However, any substances (thus chemopreventive compounds too) can induce toxicity into the body. What is more, the need for a continuous treatment over an extended period of time heightens the risk of toxicity from compounds used for chemoprevention.

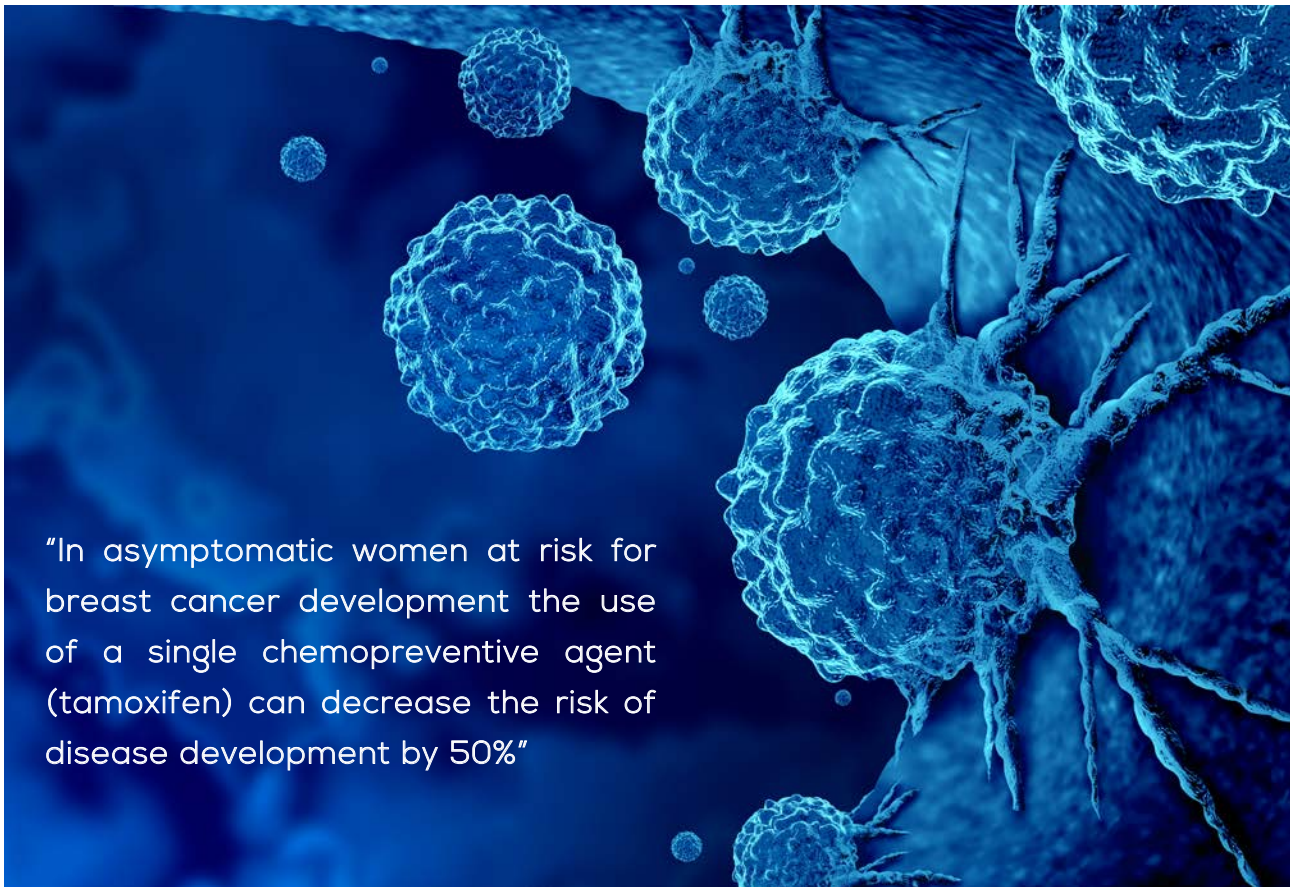
Concerns of long-term side effects make efficient chemopreventive drugs, such as tamoxifen and aspirin, are still underused. For example, tamoxifen is associated with increased risk of stroke, pulmonary embolism, and deep vein thrombosis, cataract, endometrial cancer, and vasomotor symptoms. AIs have a more favorable side effect profile than SERMs. Nowadays, for all of these chemopreventive drugs, it is recommended to offer the treatment only to women at low risk for adverse side effects.

The **repositioning of old drugs to chemoprevention** could aid reduce the concern on the adverse risks that are associated with current approved chemopreventive agent use. For example, metformin could be a viable heart-healthy alternative to tamoxifen in breast cancer chemoprevention.

Type II diabetes increases breast cancer risk. In particular, the hormone insulin, whose action is altered in diabetes, activates signaling pathways that drive aggressive breast cancer and predict poor breast cancer survival. Metformin lowers circulating insulin; moreover, it induces energetic stress in cancer cells. Ongoing primary prevention trials are evaluating its efficiency in counteracting breast cancer development.

Melatonin, the hormone naturally secreted by the human body to control the sleep/wake rhythm, could be another valuable option for women eligible for tamoxifen-based chemopreventive treatment. Among other functions, melatonin acts both as a SERM (like tamoxifen and raloxifene) and as a selective estrogen enzyme modulator (like AIs). Experimental data suggest that it could be used to enhance the effects of SERMs and AIs, and to reduce or even suppress their side effects. Also, the association of melatonin with hormone replacement therapy (which correlates with an increased breast cancer risk), could decrease the incidence of mammary cancer.

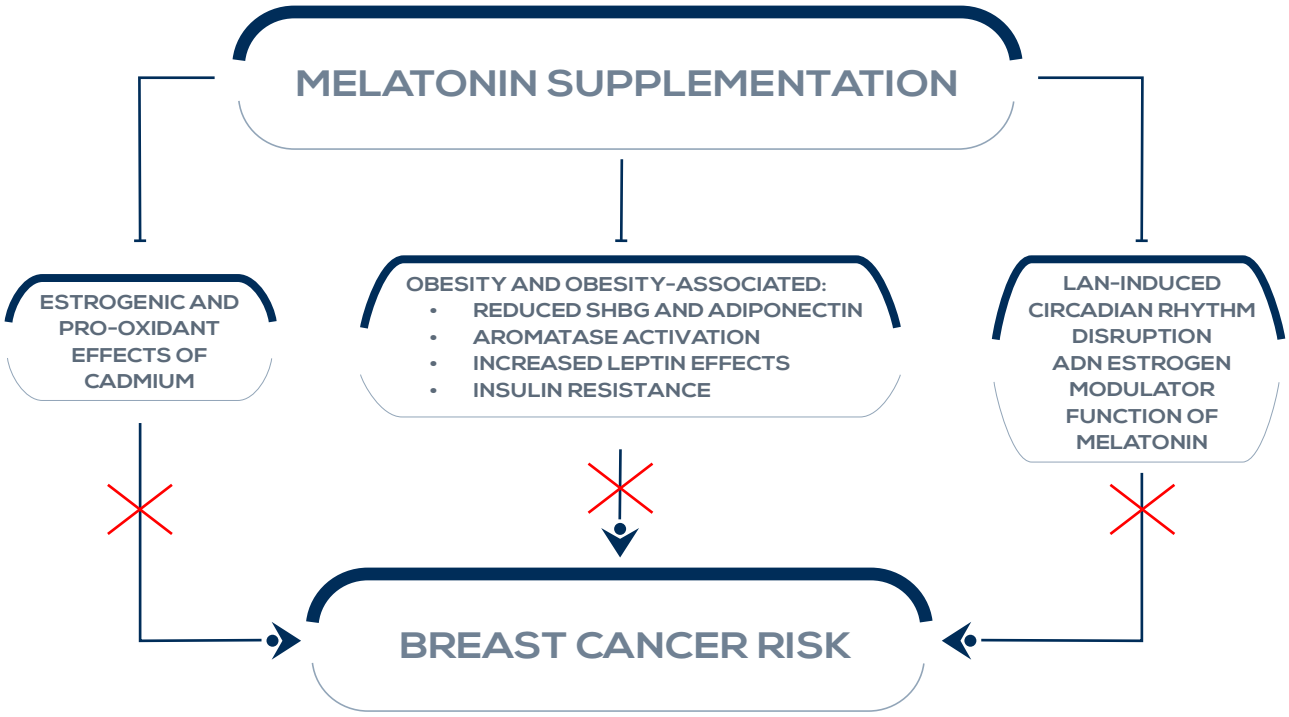
Also, melatonin could help fight cancer in several other ways. First, it is a radical scavenger potentially useful in preventing DNA damage. Second, it limits the cellular uptake of linoleic acid, an omega 6 fatty acids that promotes cancer cell proliferation. Third, melatonin inhibits telomerase, which is usually upregulated in cancer cells, contributing to their immortalization. Finally, it possesses anti-inflammatory properties, activates tumor-suppressor factors such as p53, and reduces the levels of endothelin-1, a molecule that, among other functions, protects cancer cells from apoptosis.



Melatonin's potential as a chemopreventive agent sounds particularly interesting for specific group of high-risk people, such as women at high risk of breast cancer development because of night shift work, exposure to chemical contaminants (such as xenoestrogens, that is environmental contaminants with properties similar to estrogens), or obesity. The circadian clock modulates the rhythm in processes such as redox regulation, autophagy, DNA damage repair, cellular metabolism, immune function, and inflammation; by altering these processes, disruption of circadian rhythms create a pro-tumoral environment. Moreover, the circadian clock machinery interacts with proteins involved in cancer-related pathways, and controls the expression of telomerase (the enzyme that plays a critical role in telomere length maintenance), thus influencing cancer risk. Circadian rhythm disruption by night shift work and exposure to light-at-night (LAN) contributes to cancer progression. Cell division is impacted, and melatonin secretion is inhibited. The SERM and selective estrogen enzyme modulator activities of this hormone are eliminated, leading to an increased breast cancer risk. However, melatonin supplementation could restore circulating melatonin levels, regulate circadian rhythm and correct the altered expression of clock genes involved in cell cycle control, thus reducing breast cancer risk.

Cadmium stands as an example of a chemical contaminant whose action can be counteracted by melatonin. In breast cancer, it acts as a metalloestrogen, increasing the proliferation of estrogen receptor-positive breast cancer cells and the expression of genes regulated by estrogen. What is more, cadmium induces oxidative stress by reducing antioxidant defenses and by mitochondrial damage. Melatonin seems to inhibit cadmium estrogenic effect, to down-regulate the cadmium-induced expression of telomerase, to induce proteins involved in cadmium detoxification, and to protect against cadmium-induced oxidative stress.

Melatonin could help fight cancer by counteracting obesity too. This condition can be promoted by sleep deprivation, and several clinical trials demonstrated melatonin efficacy in treating it. Looking at breast cancer, obesity increases aromatase expression, stimulating estrogens synthesis, and decreases sex hormone-binding globulin (SHBG), a protein that regulates sex hormone bioavailability. Both these effects promote tumor initiation in obese women; due to its estrogen modulator activities, melatonin could help counteract them. Finally, melatonin could help counteract the reduction in adiponectin, the estrogen receptor-activating effects of increased leptin, and insulin resistance, all of which are phenomena associated with obesity.

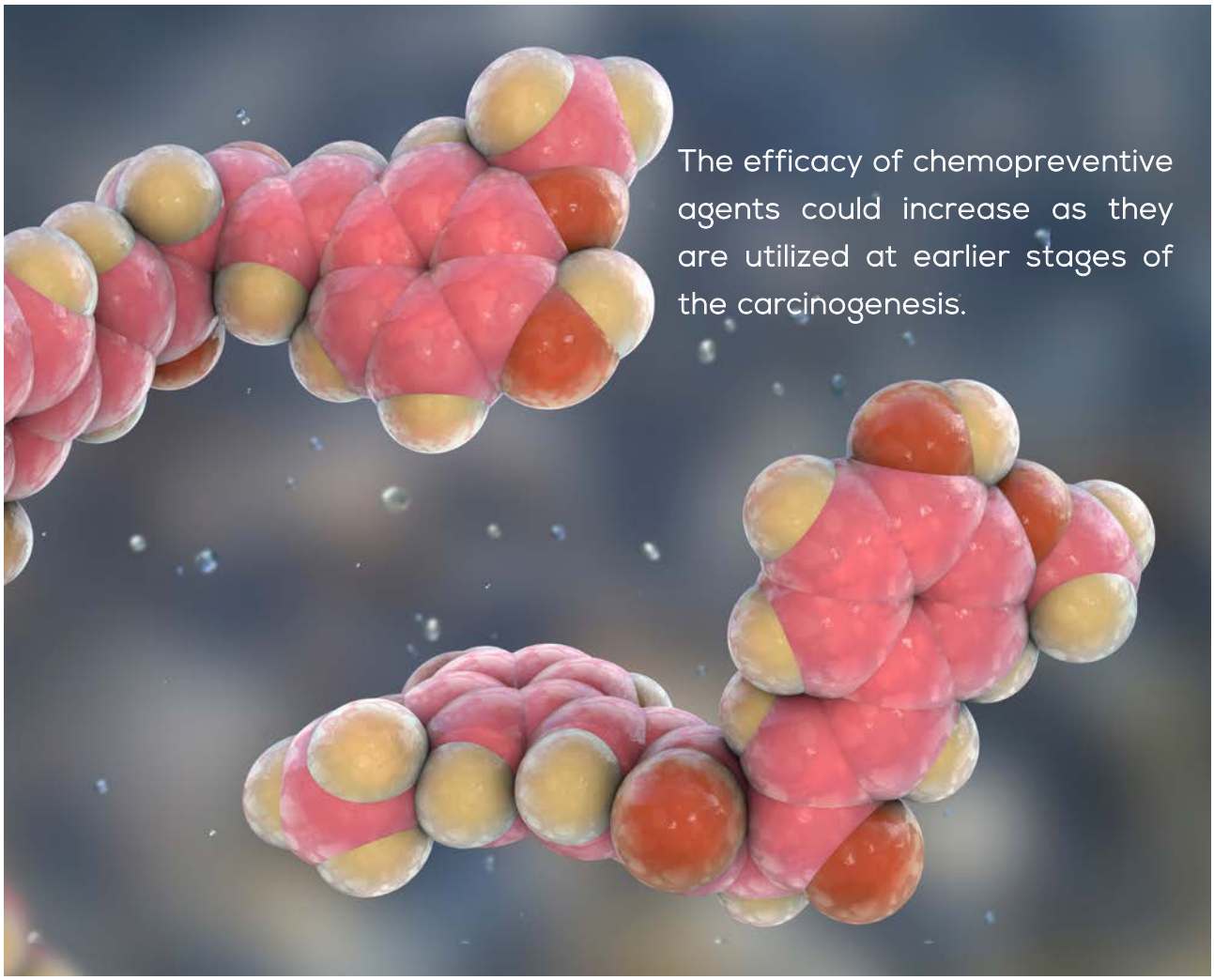


How melatonin could help fight breast cancer. LAN, light-at-night. SHBG, sex hormone-binding globulin.

Also, melatonin's potential as a chemopreventive agent goes beyond breast cancer. In fact, reduced melatonin was linked to other tumors, such as prostate and endometrial cancer. What is more, circadian rhythm disruption is deemed responsible for the increased risk of developing malignancies others than tumor of the breast, such as prostate and colorectal cancer, and in 2019 the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) confirmed the classification of night shift work in Group 2A, "probably carcinogenic to humans". Also, the IARC designates cadmium as a Group 1 human carcinogen, and several studies suggest its implication in several forms of cancer (beside breast cancer, lung, prostate, kidney, and bladder cancer). Finally, obesity is a risk factor for both breast and many other tumors, among which are colon, rectum, kidney, gallbladder, pancreas, esophageal, ovary, and uterus cancer.

In some countries, such as Italy, high-dosage melatonin (over 1 mg/day) is regulated as a drug; in others, including the USA, this hormone is considered a dietary supplement at any dosage. Melatonin supplementation presents with just minor side effects, including drowsiness, dizziness, headaches, nausea and apathy. Caution must be taken by young men, because long term administration decreases semen quality and motility; however, it could represent a valuable alternative to already approved chemopreventive drugs.

Intermittent chemoprevention could be another strategy to circumvent the adverse risks of current approved chemopreventive agent. Also, it should be noted that cancer prevention should require lower drug doses than eradicating an established tumor; thus, long-term side effects risk could be limited by **reducing the dosage for chemopreventive purposes**. What is more, toxicity could be minimized by **combining several effective chemopreventive agents at lower dose**s, reaching a double goal: to synergistically target one or more risk factors, and to reduce the risk for side effects from a single, high-dose, compound. Finally, some nutrients (vitamins, minerals, and fatty acids) **and compounds from natural sources** (in particular, plants) can act as chemopreventive agents by targeting the same objectives as currently used drugs, with a lower risk of side effects.



Berberine stands as an example of a natural alternative to chemopreventive drugs. It is the most active alkaloid in the plant *Berberis vulgaris*, which has been used for 5,000 years to treat diarrhea and dysentery in Chinese and Ayurvedic medicine. Today, it can be extracted with variable efficacy from the bark of several species, among which are *Coptis chinensis Franc* and *Hydrastis canadensis L.*

Despite different structure, berberine acts similarly to metformin, exerting hypoglycemic actions. Also, berberine possesses anticancer activity. It suppresses cell proliferation, metastasis, and angiogenesis in several cancers (including breast, lung, cervical and gastric cancer, melanoma, hepatoma, and other malignancies), performing effects similar to metformin in this case too. Also, similarly to metformin, berberine exerts anti-inflammatory actions that regulate tumor microenvironment, ameliorating cell damage and insulin resistance induced by inflammatory cytokines. Finally, it showed antioxidant and immunomodulatory properties.

In spite of such similarities, when compared to metformin, which should be used with caution in people with impaired liver or kidney functions, berberine could induce only tolerable and controllable side effects (usually, gastrointestinal discomfort), highlighting that natural alternatives to chemopreventive drugs can represent safer options.

The potential of chemopreventive agents from other vegetable products – such as soy, green tea, cruciferous plants, turmeric, tomatoes, garlic, and ginger – is highlighted by several *in vitro, in vivo* and clinical studies. Also, observational impressions and epidemiological studies demonstrate that diets richer in fruits and vegetables are associated with a lower incidence of cancer. As in the case of classical chemopreventive drugs and berberine, the anticancer activity of these products is based on the inhibition of processes such as oxidative stress, inflammation, and cell transformation, proliferation and survival. Most of them exert more than one action; however, some natural chemopreventive compounds have one or few well-established peculiar anticancer mechanisms.

Fruits and vegetables are plentiful of micronutrients and other substances deemed to counteract cancer development. Among others is **genistein**, a phytoestrogen which abounds in soybeans and soy-based foodstuffs. Genistein induces the death of precancerous breast cells. Its chemopreventive action was first suggested by the lower incidence of breast cancer reported among Asian women, which are characterized by a higher intake of soy-based foods. Following research highlighted its involvement in epigenetic reprogramming of epithelial cells, revealing that low doses of genistein are sufficient for chemoprevention.

Epigallocatechin gallate (EGCG) from green tea is potentially useful in cancer chemoprevention too. It exerts other cancer cell-suppressing actions, such as regulating specific microRNAs expression and influencing epigenetic modification of DNA. In particular, EGCG allows for the restoration of the expression of a transcription factor (RXRα) which, in cancer cells, is often downregulated by hypermethylation.

Both green tea derived molecules and **curcumin** – a long used anti-inflammatory compound from the Indian spice *Curcuma longa* (turmeric) – stabilize the inhibitors of the proinflammatory transcription factor NF-kB. What is more, curcumin can limit cell proliferation, promote apoptosis, and act as an antioxidant. It exerts a chemopreventive action based on epigenetic regulation of several genes. For example, it decreases the production of p53, a protein mutated in over 50% of cancers, resulting in apoptosis promotion.

According to a 2019 analysis, cancer is the major targeted disease in 37% of published papers on curcumin. By simultaneously affecting multiple signaling pathways (see Table 2 for some examples) it represents an extremely potent chemopreventive agent. Until now, it has been used, alone or in combination with other agents, both to prevent or treat various forms of cancer (including colorectal, pancreatic, breast, prostate, lung, and oral cancer, and multiple myeloma). For example, evidence exists of curcumin chemopreventive effectiveness when taken by patients with precancerous lesions (such as intestinal metaplasia of the stomach), and turmeric treatment was associated with a significantly reduced urinary excretion of mutagens in chronic smokers.

Curcumin is recognized as a non-mutagenic and non-genotoxic compound, and its ingestion and pharmacologic use are labeled safe by the FDA. In particular, standardized curcumin powder is safe for human use up to a dose of 1.5 grams a day and for periods up to 6 months. Adverse effects are mild, including abdominal pain, nausea, and dyspepsia.

Sulphoraphane from cruciferous vegetables such as broccoli, and **resveratrol**, the natural phenol produced by grapes and several other plants (e.g. peanuts, cranberries and blueberries), induce transcription factors responsible for the expression of free radical-scavenging enzymes. But resveratrol is mostly known for its anti-inflammatory action, which is deemed responsible for its anticancer effects.

Among phytochemicals, resveratrol showed the strongest activity similar to sirtuins, chemicals that inhibit one of the pharmacological targets of NSAIDs, cyclooxygenase-1 (COX-1). Recently, it was demonstrated that this action depends on epigenetic mechanisms too. In fact, this phenol controls the expression of several microRNAs involved in cancer development, the methylation of the promoter of tumor-associated genes, and histone modifications associated with the development of several cancers.

Human data suggest resveratrol chemopreventive efficacy in healthy people based on the significant reduction of the expression of two proteins associated with tumor formation (insulin-like growth factor 1 – IGF1 – and IGF-binding protein 3 – IGFBP3), with

only mild to moderate gastrointestinal symptoms at doses of 2.5 or more grams per day. Also, resveratrol supplementation was shown to modulate enzymes involved in the activation or in the detoxification of carcinogens; this could be one of the chemopreventive mechanisms by which resveratrol protects from cancer development.

The potential side effects of resveratrol (in particular, potential drug interactions) can be avoided by adjusting the supplementation dosage.

Anticancer molecules can be found in foods of animal origin too. In particular, fish is a source of **omega-3 fatty acids** readily available to exert an anti-inflammatory function – that, given the role played by inflammation in the pathogenesis of several malignancies (such as colorectal, gastric, and esophageal cancer), is a pillar of cancer chemoprevention. In particular, acting on the same pathway targeted

Table 2. Examples of molecular targets of curcumin in cancer.

CANCER	MOLECULAR TARGETS	EFFECT
Prostate	*Bcl-2 L1, Bcl-2 L11, BAK1, BAX, BBC3, PMAIP 1, p53*	⋀
	NFKBIA, AKT 1, Bcl-2, BIRC4, BIRC5, PTEN, NKX 3A, CSF 1R, EGFR, NF-kB	⋁
	caspase-3, caspase-8	⋀⋁
Pancreatic	*caspase-3, PARP, P-ERK1/2, c-Jun, p38 MAPK, p53, miR-200*	⋀
	NF-kB, cyclin-D1, c-myc, Bcl-2, Bcl-xL, cIAP-1, MMP, COX-2, VEGF, Sp-1, Sp-3, Sp-4, survivin, PGE_2, miR-21	⋁
Colorectal	*DR-5, IGF-1R, IGFBP-3*	⋀
	COX-2, NF-kB, Bcl-2, Bcl-xL, c-myc, VEGF, IL-8, MMP-9, PGE_2	⋁
	EGFR	⋀⋁
Breast	*TIMP-1, p21, p27*	⋀
	NF-kB, AP-1, COX-1, COX-2, VEGF, FGF, cyclin E, IL-6, IL-11, TGF-β, MMP-2, MMP-9, MMP-13	⋁
Multiple myeloma	*caspase-7, caspase-9, PARP*	⋀
	IkBα, Bcl2, Bcl-xL, cyclin D1, IL-6, COX-2, NF-κB	⋁
Leukemia	*BAX, caspase-3, caspase-8, p21, p27*	⋀
	Bcl-2, PARP, cyclin D3, STAT3, AKT, NF-κB, Mcl-1, XIAP	⋁

Adapted from: Devassy JG et al. Nutr Rev. 2015 Mar;73(3):155-65.

by aspirin and celecoxib (that is, on cyclooxygenase-2 – COX-2 – activity), omega-3 could represent a safer, natural alternative to these drugs.

Diets that are poor in omega-3 fatty acids (with higher omega-6 to omega-3 ratio) are associated with higher incidences of inflammatory diseases, including cancer. This association lies at least in part on the conversion of omega-6 in potent molecules responsible for proinflammatory functions by pro-tumorigenic enzymes that have been reported to be upregulated in cancer. Competing for the same pathways used by omega-6 fatty acids, omega-3 are instead converted into much weaker inflammatory molecules, and can even exert antiinflammatory effects. What is more, omega-3 fatty acids inhibit the activation and the expression of the proinflammatory, tumor-promoting transcription factor NF-kB, reducing the expression of proinflammatory molecules such as tumor necrosis factor-α (TNF-α). Finally, they can be converted into specialized pro-resolving mediators (SPMs, namely resolvins, protectins and maresins), powerful mediators of the resolution of inflammation. In particular, SPMs reduce inflammatory molecule production and control inflammation-related immune cell migration. these phenomena, together with a number of other mechanisms such as apoptosis and autophagy induction, angiogenesis inhibition and direct control of tumor cell proliferation, allow omega-3 fatty acids to affect tumorigenesis and cancer prevention and development.

Several human studies support the role of dietary omega-3 (in particular, eicosapentanoic acid – EPA – and docosahexanoic acid – DHA) in cancer risk reduction. For example, the use of fish oil (a rich source of EPA and DHA) was associated with reduced breast cancer risk. Also, dietary omega-3 and omega-3 supplements were associated with reduced colorectal cancer incidence, and fish oil supplementation was associated with reduced rectal mucosa cell proliferation of people with colorectal adenomas. In a randomized trial, omega-3 supplementation significantly reduced adenomas incidence in people with familial adenomatous polyposis (FAP), a very strong colorectal cancer risk factor. Finally, like aspirin, EPA showed chemopreventive activity on colorectal adenoma total burden.

Evidence of the omega-3 chemopreventive action exists for esophageal cancer too. In fact, high omega-3 intake could halve the risk of developing Barrett's esophagus (a condition associated with esophageal cancer development), and the dietary supplementation with 1.5 grams a day of EPA for 6 months significantly reduced COX-2 expression in Barrett's mucosa.

Also, **probiotics** (that is, live

microorganisms providing host health benefits) were first associated with reduced cancer incidence (in particular, a 37% reduction in colorectal cancer) more than 40 years ago. Several *in vitro* and *in vivo* studies suggest their ability to modulate cancer cell proliferation and apoptosis, and the intake of yogurt (which is naturally rich in probiotics) was associated with a decreased colon cancer risk. Even if their specific mechanism of action remains unclear, their engagement in pathways playing a central role in cancer development is widely recognized.

First, probiotics counteracts environmental conditions that affect colon carcinogenesis, in particular pH levels, the effect of bile acids in feces, the proliferation of putrefactive microbes that are involved in the production of carcinogenic compounds (such as *Escherichia coli* and *Clostridium perfringens*), and, more in general, dysbiosis (disturbances of the intestinal microbiota balance that can cause chronic inflammation and, consequently, increased cancer risk).

Both *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, well-known probiotic species, modulate colonic pH and bile acid profile. In colon cancer patients, a 6-weeks L. *acidophilus* supplementation was associated with reduced *E. coli* and *Clostridium* spp., and probiotics' ability to reduce the levels of bacterial harmful enzymes was confirmed by human studies. For example, the oral supplementation of *Lactobacillus acidophilus* strain N-2 or NCFM promoted a significant

Table 3. Evidence of omega-3 supplementation chemopreventive effects on colorectal cancer development.

REFERENCE	METHOD	POPULATION	DOSE	DURATION	RESULTS
Kantor et al., 2014	Fish oil supplements vs no use	US adults	nd	4+days/week for 3+years	Reduced colorectal cancer risk
Aglago et al., 2020	Highest intake vs lowest intake	European adults	> 470 mg/day	Median 14.9 years	Reduced colorectal cancer risk
West et al., 2010	EPA-free fatty acids	FAP patients	2 g/day	6 months	Reduced polyp diameters

2- to 4-fold reduction in the activity of azoreductase, nitroreductase and β-glucuronidase in all the healthy people involved in the test. *Bifidobacterium* sp. fermented milk was associated with reduced β-glucuronidase activity, and *Lactobacillus* GG, supplemented with or without rye fiber, was associated with significantly reduced β-glucuronidase and nitroreductase.

Second, probiotics can bind to and degrade potential carcinogens, such as mutagenic compounds commonly found in food. Human studies showed that *L. acidophilus* and *L. casei* supplementation helps lower the mutagenic potential of, respectively, cooked meat-rich diets and fried ground meat.

Third, probiotics can affect carcinogenesis by modulating the activity of the immune system. In particular, they can influence the production of molecules involved in inflammation (cytokines and prostaglandins) and activate immune cells (phagocytes) responsible for the direct elimination of early-stage cancer cells. The ingestion of fermented milk containing *L. casei* Shirota was associated with increased activity of NK cells, immune system cells that can kill tumor cells.

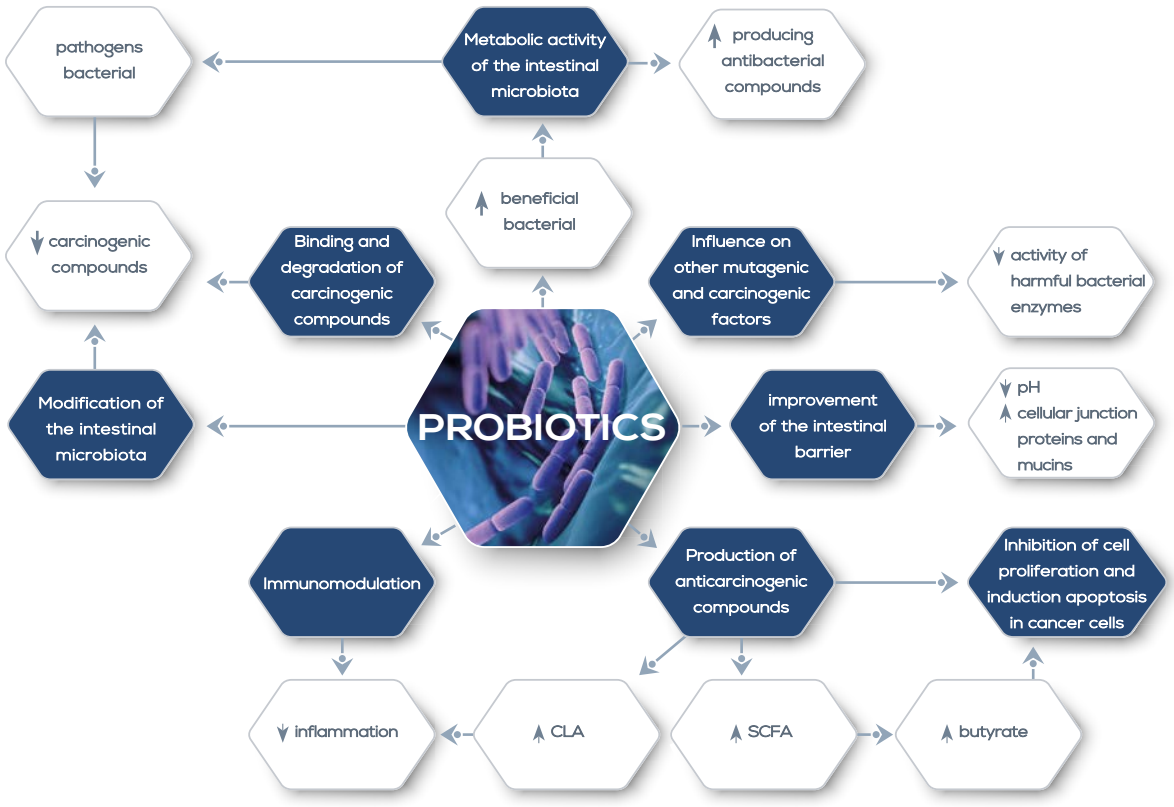
Finally, probiotics improve the intestinal barrier by influencing the mucus layer and the distribution of cell junction proteins, and certain strains of *Bifidobacteria* and Lactobacilli can produce conjugated linoleic acids (CLAs) or affect the production of short-chain fatty acids (SCFAs). CLAs influence apoptosis, the cellular response to growth factors, and the production of molecules involved in the inflammation process. SCFAs are signaling molecules that affect, among others, immune system, inflammation, cell death and proliferation; in particular, butyrate promotes apoptosis, inhibits proliferation of colon and cultured cancer cells, decreases the production of inflammatory cytokines, and improves the intestinal barrier by increasing mucus production (reduced in carcinogenesis) and by promoting the proliferation of healthy cells. Decreased SCFAs and SCFA-producing bacteria dysbiosis are strongly associated with colorectal cancer, and in animal models the administration of butyrate-producing bacteria (*Butyrivibrio fibrisolvens* MDT-1) inhibits cancer development, affects mutagenic enzyme activity, and increases the immune responses. Even if usually not directly involved in the production of short-chain fatty acids, probiotics can influence SCFA production by modulating gut microbiota composition. In particular, preclinical research suggests that the combination of probiotics and prebiotics (that is, substances that promote probiotics growth) could help counteract colorectal cancer by increasing SCFA-producing bacteria.

It is noteworthy that dysbiosis may promote cancer development outside the gastrointestinal system, and that prebiotics can also counteract cancers other than colorectal. In particular, the use of probiotics-based supplements increases the eradication of *Helicobacter pylori*, whose infections are associated with gastric cancer, and the clearance of human papillomavirus (HPV)-related cervical cancer precursor lesions. Nowadays, many clinical studies suggest that probiotics could be effective in preventing several types of cancer, including colorectal, breast, cervical, bladder and liver cancer.

The detection of significant differences

between the gut microbiota from healthy people and from patients with polyps (small clumps of cells that can develop into colon cancer) suggests that the intestinal flora is involved in cancer development since its very early stage. This makes probiotics ideal potential chemopreventive agents.

By far, *Lactobacillus* and *Bifidobacterium* (that is, lactic acid-producing bacteria) have been among the most studied genera of probiotics potentially useful against cancer development. However, next-generation probiotics are facing the field of cancer chemoprevention. For instance, *Butyricicoccus pullicaecorum*, a butyrate-producing species belonging to the *Clostridia* class, is emerging as a safe alternative to classical probiotics targeted at countering disease-associated gut flora alteration by increasing butyrate production.



Potential mechanisms of probiotics in colorectal cancer chemoprevention.

Adapted from: Śliżewska K et al. The Role of Probiotics in Cancer Prevention. Cancers (Basel). 2021 Jan; 13(1): 20.

Table 4. Results of clinical studies evaluating probiotics effectiveness in cancer prevention.
Adapted and modified from: Śliżewska K et al. The Role of Probiotics in Cancer Prevention. Cancers (Basel). 2021 Jan; 13(1): 20.

REFERENCE	POPULATION	PROBIOTICS	DURATION	RESULTS
Ohara T and Suzutani T, 2018	Healthy men, average age 60.2 years	*Bifidobacterium longum* (BB536-y) alone or in combination with fructo-oligosaccharides (FOS)	5 weeks	Increased total fecal SCFAs and reduced *Bacteroides fragilis* enterotoxin and the growth of putrefactive bacteria
Toi M et al.,2013	Breast cancer patients aged 40-55 years	*Lactobacillus casei* Shirota + isoflavones from soy products	2 years	Consuming probiotics + isoflavones since adolescence correlates with reduced breast cancer incidence
Pala V et al., 2011	Healthy men and women	*Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus*	12 years	Increased consumption of yogurt correlates with reduced colorectal cancer risk (especially in men)
Ohara T et al., 2010	Colorectal cancer patients vs healthy patients	*Lactobacillus gasseri* OLL2716 (LG21)	12 weeks	Increased *Lactobacilli* and immune cell activity, reduced *Clostridium perfringens*
Ma EL et al., 2010	Women with HPV-positive intra epithelial lesion	*Lactobacillus casei* Shirota	6 months	60% reduction in HPV-associated infections and cervical cancer precursors
Hatakka K et al.,2008	Men aged 24-55 years	*Lactobacillus rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *Shermanii* JS	4 weeks	Reduced mutagenic enzyme activity, increased *Lactobacilli* and *Proprionibacteria*
El-Nezami HS et al., 2006	Male students with high urine aflatoxin level	*Lactobacillus rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *Shermanii*	5 weeks	61.5% reduction in a liver cancer biomarker which leads to reduced aflatoxin B1-N7guanine urinary excretion
Ohashi Y et al., 2002	Bladder cancer patients vs population-based controls	*Lactobacillus acidophilus* L1	10 weeks	Habitual intake of lactic acid bacteria correlates with reduced bladder cancer risk

CHEMOPREVENTIVE TREATMENT TARGET POPULATIONS

Everybody should start to prevent cancer from a young age. That means everybody should follow the principles of primary prevention such as avoid smoking and elevated alcohol consumption, eat a healthy and balanced diet, and protect himself from air pollution and sunburns. What is more, some people should think about undergoing a chemopreventive program to reduce their elevated risk of cancer development.

For example, according to the FDA, women at least 35 years old with a 5-year predicted risk of breast cancer ≥ 1.67% (calculated by the Gail model for breast cancer risk) are eligible for a tamoxifen-based chemopreventive treatment. Similarly, postmenopausal women with at least one breast biopsy that showed lobular carcinoma *in situ* or atypical hyperplasia, breast cancer in one or more first-degree relatives, or a 5-year predicted risk of breast cancer ≥ 1.66% (calculated by the modified Gail model) are eligible for raloxifene-based chemopreventive treatment.

Aspirin or celecoxib treatment is suggested to prevent colorectal cancer, respectively, in adults aged 50-59 years with a 10% or greater 10-year cardiovascular risk, no increased risk for bleeding, a life expectancy of at least 10 years, and willing to take low-dose aspirin daily for at least 10 years, or adults aged ≥ 18 years with familial adenomatous polyposis.

In people with actinic keratoses, skin cancer can be prevented by fluorouracil, 3% diclofenac sodium, combined 5-aminolevulinic acid and photodynamic therapy (in case of actinic keratoses of the face or scalp), imiquimod (in immunocompetent people with actinic keratoses of the face or scalp), or ingenol mebutate (in case of actinic keratoses on the face, scalp, trunk and extremities).

Also, people with high-grade dysplasia in Barrett's esophagus can rely on porfimer sodium plus photodynamic therapy and omeprazole to prevent esophageal cancer, whereas males and females with carcinoma *in situ* (CIS) of the urinary bladder can reduce bladder cancer risk with the Bacillus Calmette-Guérin (BCG, the live attenuated strain of *Mycobacterium bovis* that is utilized in both CIS treatment and recurrence risk reduction) or, if refractory to BCG, valrubicin.

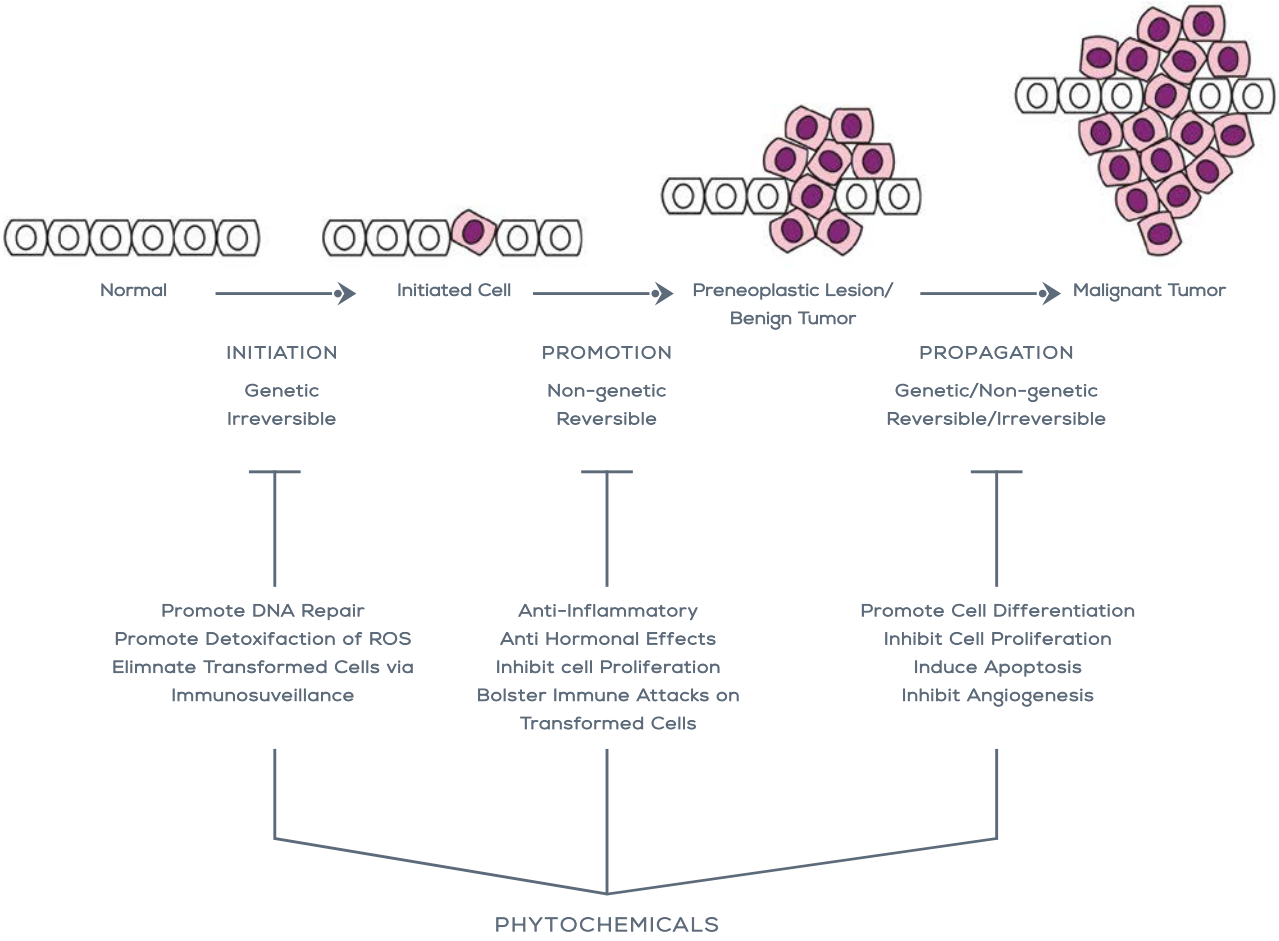
Nowadays, it is possible to take advantage of the newest molecular approaches to cancer interception to counteract disease development by **starting a chemopreventive program based on the interception of asymptomatic conditions that contribute to increased cancer risk**. In particular, the analysis of the cell-free DNA (cfDNA) circulating in the blood allows for intercepting genomic instability, that is the accumulation of genetic and epigenetic changes that can lead to cancer development. What is more, it is possible to intercept cancer driver conditions that can promote genomic instability or insist on it (chronic inflammation, immune system imbalance, an altered microbiota, and shorter or longer telomeres). The presence of one or more of these conditions results in the amplification of the risk of cancer development.

Once intercepted, both genomic instability and the other cancer driver conditions can be actively counteracted by the use of chemopreventive agents. Nutrients, other natural compounds and probiotics can be utilized at non-toxic dosage to help cells cope with them and hinder cancer development at any age.

DIETARY PHYTOCHEMICALS AND CANCER CHEMOPREVENTION

carcinogenesis is a multistep process that ultimately reprogram a normal cell into a cancer cell.

Adapted from: Śliżewska K Phytochemicals may exert their chemopreventive effects by blocking key events of tumor initiation and promotion thus reversing the premalignant stage. These agents may also prevent tumorigenesis by inhibiting or retarding tumor progression or by promoting cell differentiation.



Adapted from: Dietary phytochemicals and cancer chemoprevention: a review of the clinical evidence; R Kotecha, A Takami and J Espinoza

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