UNIVERSITÀ DEGLI STUDI di MILANO

DIPARTIMENTO di SCIENZE FARMACOLOGICHE E BIOMOLECOLARI
CORSO di DOTTORATO in SCIENZE FARMACOLOGICHE SPERIMENTALI E CLINICHE
XXXIII Ciclo

PhD Thesis

THE ANTITUMORAL ROLE OF HDL: IN VITRO STUDIES ON PROSTATE CANCER CELL LINES

PhD candidate:
Eleonora Giorgio
R11915

Supervisor: Prof. Monica Gomaraschi
Coordinator: Prof. Alberico Luigi Catapano

Academic Year 2019-2020

Sometimes you win,

Sometimes you learn.

Index

Abstract

Background - Prostate cancer (PCa) is the most common malignancy in men. After initial response to androgen deprivation therapy, PCa can evolve to a castration-resistant phenotype, for which therapeutic options are poorly effective. In the tumor microenvironment, sources of cholesterol, reactive oxygen species (ROS) and pro-inflammatory molecules can favor cancer cell proliferation. Indeed, cells need cholesterol to proliferate, as structural and functional component of cell membranes and as substrate for the synthesis of hormones and oxysterols. In addition, recent evidence suggests that oxidative stress can play a role in the pathogenesis and the progression of prostate cancer (PCa).

Aim - Since HDL are known to promote cell cholesterol efflux and to exert antioxidant activities, aim of the study was to investigate whether HDL can reduce cholesterol content and oxidative stress in PCa cells, thus modulating their proliferation.

Methods - Androgen-sensitive and -resistant PCa cell lines (LNCaP and PC-3, respectively) were tested and compared with non tumor prostate epithelial cells (PNT2). Lipoproteins were isolated by ultracentrifugation from the plasma of healthy volunteers. ROS levels and cell cholesterol content were assessed by fluorescence. Oxidative stress was induced by H_2O_2 and cell cholesterol content was increased by incubation with LDL. The expression of proteins involved in cell cholesterol metabolism was assessed by by RT-PCR, western blotting or immunofluorescence. Cell proliferation was assessed by cell count, cell cycle analysis and colony formation assay. Bortezomib was used to inhibit the proteasome.

Results - HDL significantly reduced basal and H_2O_2-induced oxidative stress in normal, androgen-sensitive and -resistant PCa cell lines. The androgen receptor, SRBI, ABCA1 and G1 transporters were not involved. Consequently, HDL completely blunted H_2O_2-induced increase of cell proliferation, through their capacity to prevent the H_2O_2-induced shift of cell cycle distribution towards G2/M phase. Cholesterol homeostasis is altered in PCa cells. PCa cells, that needs of cholesterol, mainly rely on lipoprotein uptake than intracellular synthesis of it. In LNCaP cells, the incubation with LDL increased cholesterol content and cell proliferation. The concomitant incubation with HDL promoted cell cholesterol reduction and blunted LDL-induced cell proliferation. On the contrary, in PC-3 cells HDL caused a further increase of cholesterol content and lost their ability to limit LDL-induced cell proliferation. In PC-3 cells, ABCA1 mRNA levels were elevated, but the protein was hardly detectable in the absence of pathogenic mutations in the ABCA1 gene. The treatment with the proteasome inhibitor bortezomib restored ABCA1 expression and consequently the ability of HDL to reduce cell cholesterol content and LDL-induced proliferation.

Conclusions - Collectively, HDL can inhibit the proliferation of androgen-sensitive and castration-resistant PCa cell lines induced by oxidative stress and LDL. In castration-resistant PCa cells, HDL prevented LDL-induced cell proliferation only after the restoration of ABCA1 expression through proteasome inhibition.

Introduction

Prostate

The prostate is a gland of the male genital apparatus, located in the pelvis below the bladder and in front of the rectum. It is crossed by the ejaculators ducts that result in the prostate urethra; the first section of the urethral canal surrounded by the prostate itself. It has a similar size and morphology of a chestnut. Its shape is kept stable throughout the life of the individual, while the size varies. In the child it has a reduced size, and then develops during puberty, until it reaches, under physiological conditions, a weight between 10 and 20 grams *(Hricak et al., 2009)*. It is formed by three types of tissue, each of which performs a different function: (i) smooth muscle tissue, allowing the gland to contract, regulating urinary flow and contributing to ejaculation; (ii) fibrous tissue, gives support and coats it forming a capsule that isolates it from adjacent organs; (iii) exocrine glandular tissue, specialized in the production of seminal fluid, contributes to 20-30% of the total volume of ejaculate, joining sperm from the testicles and other fluids produced by seminal vesicles from sperm to sperm. Today the nomenclature most used to describe it is that of McNeal *(Hodge et al., 1989)*, whereas in the past lobes were used. McNeal proposed subdivision of the prostate into anatomically separate zones (Figure 1), which differ in histology and biological behaviour *(McNeal 1980)*: (i) the transition zone includes only 5% -10% of parenchyma tissue. It consists of two small lobes placed laterally in the urethra in the median area. It is involved in the development of benign prostate hyperplasia while the development of adenocarcinoma is less common. Its periurethral position denote how modest nodal enlargements can produce obstructive symptoms. (ii) The central area includes 20% of the prostate parenchyma. It is the richest area of glandular elements. It is cone-shaped and surrounds the ejaculated ducts. It is the least area affected by disease. (iii) The peripheral area represents 70% of the gland. It is the part detected during the rectal palpation examination. And it is the area most susceptible to the development of carcinomas and inflammatory processes. (iv) The fibromuscular stroma is located at the front of the gland. It probably has the function of contraction during ejaculation by impending the retrograde flow of the secret.

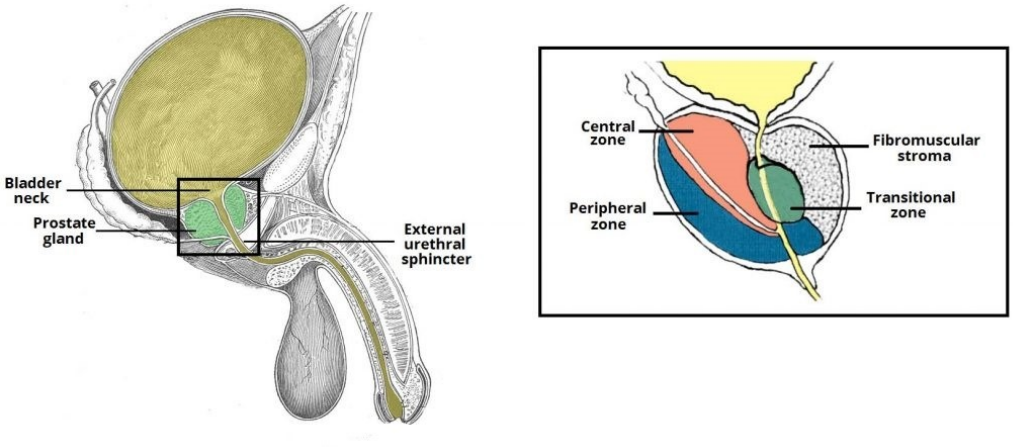


Figure 1. Anatomical position and zones of the prostate.

The prostate acts in the synthesis and secretion of the main portion of the seminal fluid, called prostate fluid, which at the time of ejaculation is transferred into the urethra, and added to other secrets. The prostate fluid is a dense and milky fluid, composed of simple sugars (fructose, glucose), enzymes and alkaline or basic compounds. Sugars feed sperm and promote their movement towards ovary. Alkaline compounds neutralize acid vaginal secretions that can damage sperm. Additional important component of the seminal fluid is PSA (specific prostate antigen), which fluidize the sperm accumulation thus allowing the movement of sperm in the female genital tract. Another function of this organ is the urinary function, in fact urinary movement is regulated by two valves or sphincters. The internal urethral sphincter is located at the entry point of the prostate, while the external urethral sphincter is placed inferiorly, at the exit point of the prostate. The muscle tissue of the prostate acts as a collar around the urethra: if there are contract of the muscle part the urine flow stops and if there are muscle relax the flow resumes.

The prostate consists of about thirty tubule-alveolar glands, coated with a muscle-connective stroma in continuity with the capsule that coats the organ. The glands are contained in lodges created by fibrous septum that depart from the urethra to inside the organ. The glands converge with their excretory ducts towards the urethra where they pour their secret *(Ambrosi et al., 2006)*. Each of them is made up of a branched excretory channel with alveolar and tubular adhesions coated with secretory epithelium. This epithelium consists of a basal layer, responsible for cell proliferation, and by one called secretory layer, responsible to produce different proteins such as metal-proteases and serine-proteases or prostate antigen (PSA). The prostate cell population consists of stromal and epithelial cells,

but also neuroendocrine cells spread across the peripheral area, prostate ducts, urethra and bladder neck region.

The prostate is mainly vascularised by lower bladder arteries, rectal arteries and internal pudenda. The veins form a rich plexus which discharges into the periprostatic one affluent of the internal iliac veins. Lymphatic vessels are in the internal iliac lymph nodes and nerves derive from the hypogastric plexus *(Ambrosi et al., 2006)*.

The prostate is an androgen-dependent organ, its growth and function depend on male sex hormones, androgens. The processes that stimulate or inhibit the release of hormones depend on the hypothalamus-hypophyses-gonadic axis. The testes, the male gonads, produce steroid hormones following stimulation by the gonadotropic hormones FSH and LH produced by the hypophyses, stimulated in sequence by the hormone GnRH released by the hypothalamus. Plasma levels of LH and FSH are low until puberty. The prostate gland remains elementary until puberty when, under the influence of increased levels of circulating androgens, begins to develop. Testosterone is a hormone produced by Leydig's testicular cells and its synthesis is controlled by a pituitary hormone, the luteinizing hormone (LH), which is in turn regulated by a hypothalamic release hormone, LHRH. At the hypothalamic level, testosterone exerts the product-inhibition mechanism that allows you to maintain testosterone levels at physiological conditions (10-35 g/L). A smaller source of androgens is the adrenal cortex where, under the influence of adrenocorticotropic hormone (ACTH), androstenedione is released from the glands. When the testicular function is intact, this supplementary androgenic activity is essential for the survival of some androgen-dependent cell clones in prostate cancer after chemical or physical castration *(Crawford et al., 1989)*. Once in the prostate, both testosterone and adrenal androgens are metabolized by the 5α-reductase to DHT, which has a ten times affinity greater for androgen receptors *(Smith et al., 1996)*. These receptors, once they enter the nucleus, bind to specific DNA sequences by regulating the expression of many genes involved in growth and cell proliferation, maintaining prostate homeostasis *(Bruchovsky et al., 1975)*.

Functional alteration

The prostate is affected by several pathologies; the most important are inflammation (acute and chronic prostatitis), hyperplasia and carcinoma. They can occur starting from puberty, when the prostate gland undergoes a volumetric increase and begins its secretory activity *(Pisani et al., 1990)*. Acute prostatitis is usually caused by lower urinary tract infections or represents a complication of urethral catheterization. Benign prostate hyperplasia (BPH), or prostate adenomatosis, consists in an enlarged and it is often wrongly considered a prelude to the onset of cancer. It is a general phenomenon, especially with advancing age, in which androgen are not produced by androgenic hypersecretion: it is believed that at the base there is testosterone conversion into his active DHT metabolite via enzyme 5α-reductase or excessive use of the DHT itself. It is also possible that the estrogens, which increase with age, also increase the expression of androgen receptors developing the pathogenesis of the disease *(Celotti., 2006)*. The treatment of choice for BPH involves the use of 5α-reductase inhibitors, such as finasteride and dutasteride, which block the action of the DHT on the gland by inhibiting its enlargement and sometimes volume; other drugs are phyto-therapeutic, against the inflammatory process, and α1-adrenergic antagonists that relax the muscles of the urethra. If these therapies are not efficacy, it is possible the intervention with surgery or destruction of adenomatous tissue by laser.

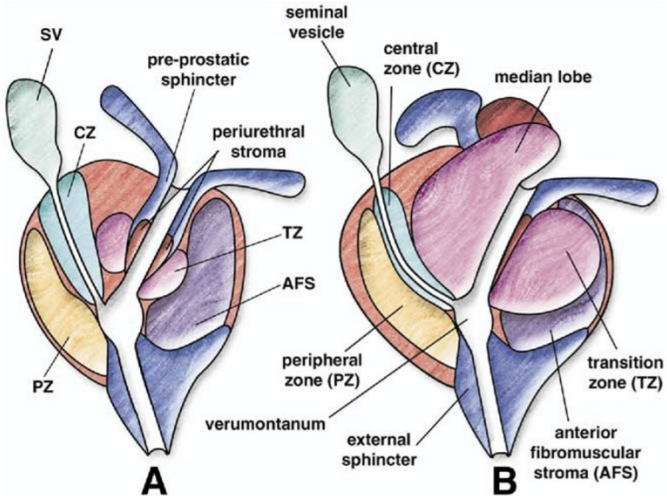


Figure 2. Zonal anatomy of the prostate. (A) Young male with minimal transition zone hypertrophy. Note preprostatic sphincter and peri-ejaculatory duct zone (central zone of McLean) are clearly defined. (B) Older male with transition zone hypertrophy, which effaces the preprostatic sphincter and compresses the periejaculatory duct zone. AFS = anterior fibromuscular stroma; CZ = central zone; PZ = peripheral zone; SV = seminal vesicle; TZ = transition zone.

Prostate cancer (PCa) is one of the most common malignant cancer diseases in male population, to account for about 20% of all cancers diagnosed in Italy *(AIOM-AIRTUM., 2018)*. Survival of patients with PCa is currently 91% from 5 years after diagnosis and is constantly growing. In 70% of cases the primitive location of adenocarcinoma is located in the peripheral zone of the gland (Figure 2) and is often appreciable with rectal exploration, 20% of cases reside in the antero-medial part of the organ transition zone (Figure 2) where the onset of the IPB, only 5% are based in the central zone (Figure 2), which, as mentioned above, is area of onset of the tumor. Cancer is mostly multifocal *(Young et al., 2000)* and symptoms are only exacerbated in the late stages of the disease, generally when there is the involvement of other organs. In particular, PCa initially develops within the gland and subsequently can spread locally by invading the seminal vesicles and the bladder neck. It can metastasize both lymphatical and haematological way and involve the bones that produce an osteoblastic response. For these reasons, when the patient presents even just a urinary disorder, especially poor and painful urination, the urologist provides with a check of the size and uniformity of the prostate through digit-rectal exploration and the prescription of PSA blood levels. In recent decades, screening programs have been activated in males over 50, to prevent the development of this cancer.

The aetiology of prostate cancer is multifactorial and depend on a complex interaction among genetic, responsible for familiarity and the different incidence in human races, and environment factors, such as diet and carcinogen in the environment.

Diagnosis and stages of prostate cancer

The diagnosis of prostate cancer is made through rectal exploration, transrectal ultrasound, prostate needle biopsy and PSA dosage. PSA, or kallikrein-3, is a glycoprotein produced mainly from glandular tissue *(Polascik et al., 1999)* and it is secreted in the seminal fluid and, only minimal amounts reach the bloodstream. This cause a malignant blood levels increase, and the antigen is considered a marker of prostate pathology. PSA is present in free form and conjugated to enzyme inhibitors or transport proteins, such as anti-chymotrypsin and alpha-2-macroglobulin *(Polascik et al., 1999)*. The immunometric methods for total PSA measure both forms, the ability to correctly measure the value is affected by the type of method used (antibodies, reference standards). The availability to

use a national standard of reference has reduced the variability between methods. However, while representing the principal test for early diagnosis, PSA is an imperfect tumor marker, because it is not very sensitive for false negatives, and not very specific for false positives, as it has also increased in the case of hyperplasia and prostatitis. The threshold value used for possible presence of PCa is 4 ng/ml. In 20-30% of cases, however, false negatives can be obtained, as the PSA levels may remain normal for some men with PCa. In contrast, 75% of men with PSA between 4.0 and 10.0 ng/ml are subjected to prostate biopsy unnecessarily, as in reality they do not have cancer *(Barry., 2001)*.

To increase diagnostic specificity, two parameters can be used: PSA "velocity", that is the PSA rate increased over time on an annual basis, and PSA "density", that is the ratio of gland size to ultrasound and circulating PSA. The periodic dosage of PSA remains the most appropriate tool to affect the disease and reduce its mortality. In cases of suspected cancer, clinical examinations could be performed: biopsy, nuclear magnetic resonance imaging (NMR) for the individuation of the extension of cancer to other organs, such as liver and lymph nodes, and BONE-SCAN, that is bone scintigraphy to detect any metastases at the bone level. The PSA can also serve to stage prostate cancer, assess relapse, the volume of cancer and the therapeutic response.

Prostate cancer is classified according to the stage, which indicates the state of the disease, and the degree, which indicates its aggressiveness. The PCa is classified into four stages based on the TNM classification, system international stage-to-stage that considers three parameters: primitive tumor size (T), involvement of regional lymph nodes adjacent to cancer (N) and the presence of metastases (M) (Figure 3) *(Cheng et al., 2012)*. Stage T1 corresponds to a tumor mass clinically not appreciable, not palpable, or visible through diagnostic imaging. This stage differs in: T_{1a} if the tumor is discovered randomly in 5% or less removed tissue, T_{1b} if it is discovered randomly in more than 5% of the tissue and T_{1c} if it is diagnosed with needle biopsy. Stage T_2 outlines a detectable tumor at palpation as a mass of variable sizes. This stage differs in: T_{2a} if the tumor affects half or less of a lobe, T_{2b} if it is more interested of the middle of a lobe but not both lobes and T_{2c} if it affects both lobes. In both T_1 and in T_2 the tumor remains intracapsular and limited to the prostate. Stages T_3 and T_4 reflect a cancer of an extra-encapsulated type, locally advanced that goes to affect adjacent organs. In T_3, the tumor reached seminal vesicles, with distinction in T_{3a} if the tumor is extra-capsular or T_{3b} if it invades the seminal vesicles. In T_4, the tumor also

invaded bladder and rectum and may have metastasized to lymph nodes and other organs. The metastatic diffusion occurs at first by lymphatic way (N), then it becomes also hematogenic (M) and involves mainly bones. N_1 indicates the presence of metastases in regional lymph nodes and with M_1 that of remote metastases.

CLASSIFICATION	DEFINITION
TUMOR	
Tx	Tumor cannot be evaluated (due to lack of information)
T0	No evidence of a primary tumor
T1*	Tumor was not detected during a digital rectal exam (DRE) and cannot be seen on imaging studies (tumor may be discoverd during surgery for a reason other cancer)*
T2 T2a T2b T2c	Tumor can be detected during a DRE but is present in the prostate only Tumor is in half or less than one side (lobe) of the prostate Tumor is in more than half of one prostate lobe, but has not yet invaded the other lobe Tumor is in both prostate lobes
T3 T3a T3b	Tumor extends outside of the prostate Tumor extends outside the prostate on one or both sides Tumor has spread to the seminal vesicles (the glands on each side of the bladder)
T4	Tumor has spread to tissues near the prostate other than the seminal vesicles, such as the bladder or wall of the plevis
Nearby (regional) lymph nodes (N)	
Nx	Nearby lymph nodes are not evaluated
N0	No cancer cells are found in nearby lymph nodes
N1	Cancer cells are found in nearby lymph nodes
Distant Metastasis (M)	
M0 M1 M1a M1b M1c	Cancer has not spread beyond the prostate Cancer has spread beyond the prostate Cancer has spread to distant lymph nodes Cancer has spread to bone Cancer has spread to another organ or site, with or without bone disease

Figure 3. A staging system is a standard method for the cancer care team to describe how far a cancer has spread. The TNM system is based on these 5 key pieces of information.

PCa should also be classified on a histological basis. The so-called "Gleason grade" is also assigned, i.e. a number between 1 and 5 indicate how similar or different the tumor glands are compared to normal glands. The final score is calculated with the sum of two stages:

the first 1 to 5 (according to the increasing anaplasia of the tumor) based on the largest area occupied by the tumor, the second by the second largest area of the tumor *(Epstein et al., 2010)*. Tumors with Gleason degree less than or equal to 6 are low grade and cancer glands still like normal, those with Gleason equal to 7 degree are intermediate, while those where it is between 8 and 10 are high-grade. It has an increased risk of progressing and spreading to other organs. The integration between these parameters (T and G) and the PSA level allows you to attribute to the three different risk classes *(Epstein et al., 2010)*:

- Low risk: T_1-T_{2a}, PSA < 10 ng/mL and Gleason score ≤ 6;
- Intermediate risk: T_{2b}-T_{2c}, PSA 10-20 ng/mL and Gleason score = 7;
- High risk: T_{3a}, PSA > 20 ng/mL e Gleason score > 7.

Nowadays there is a new prostate grading system that is an extension of the current Gleason grading scale for determining the stage of prostate cancer (Figure 4). This system is designed to provide a simplified and more accurate grading stratification system than the current Gleason Score. This new method is especially focused on better representing low grade disease to reduce unnecessary treatment of prostate cancer. The new grading system subdivides prostate cancer into five categories using pathological characteristics.

TRADITIONAL GLEASON SCORE	NEW GRADING SYSTEM GROUP 1
GLEASON 3+3=6 Only individual discrete well-formed glands	GRADE 1
GLEASON 3+4=7 Predominantly well-formed glands with a lesser component of poorly-formed/fused/cribiform glands.	GRADE 2
GLEASON 4+3=7 Predominantly poorly-formed/ fused/cribriform glands with a lesser component of well-formed glands.	GRADE 3
GLEASON 4+4=8 Only poorly-formed/fused/cribriform glands or -Predominantly well-formed glands with a lesser component lacking or -Predominantly lacking glands with a lesser component of well-formed glands.	GRADE 4
GLEASON 9-10 Lacks gland formation (or with necrosis) with or without poorly-formed/fused/cribriform gland.	GRADE 5

Figure 4. New Prostate Cancer Grading System with Gleason score.

Progression of the tumor

In the early stages of the disease, a series of carcinogenic events lead to the alteration of some cells that begin to proliferate uncontrollably. Androgens are essential to the survival and proliferation of these cells. If the tumor is discovered in time, is possible begin a drug castration therapy, with anti-androgens, or remove with surgical intervention the testicles, which are the largest producers of androgens. Androgenic deprivation therapy (ADT) works by lowering testosterone and DHT or interfering with their binding to the androgen (AR) receptor, one of the main therapeutic targets of PCa *(Gao et al., 2013)*. This therapy is very effective at destroying androgen-dependent cancer cells; but some time some cells start to survive even in androgen deprivation conditions, developing an androgen-independent phenotype, the so called castration-resistant cancer (CRPC). The development of these resistant cells is associated with an increase in angiogenesis and an increase in metastasis especially at the level of lymph nodes and bones. There are several possible androgen-

independence progression mechanisms, these include intra-tumoral synthesis of androgens, AR-gene amplification, AR-activating mutations, or AR activation through growth factors *(Gao et al., 2013)*. The hypothesis of intra-tumoral synthesis of androgens in CRPC is that there are intraprostatic residues of testosterone and DHT even after androgenic deprivation. These levels are enough to induce cancer progression *(Armadari et al., 2014)*. AR is overexpressed or mutated in many cases of PCa. The androgen phenotype can be carried out with different mechanisms:

a) Overexpression of the AR gene: some studies have found that 25-30% of androgen prostate cancers have a hyperexpression of the AR coding gene *(Koivisto et al., 1997)*, promoting the survival of cancer cells in a poor androgen's environment. The amplification of the AR gene occurs only in the tumors of patients whose disease has progressed during treatment, and among them occurs in those who initially responded effectively to therapy. This suggests that AR amplification is a mechanism of resistance acquired to the therapy.

b) Mutation of the AR gene: the AR receptor has a terminal N-domain (NTD) that influences the efficiency central DNA-binding domain (DBD), which binds to a specific element of the hormone response and determines the specificity for the target gene, and finally a C-terminal domain that binds the hormone (LBD) (Figure 5). Full or partial deletion of the N-terminal domain NTD or central domain DBD makes AR inactive in transcription, although it is still able to bind androgens with high affinity. The AR gene is located on the X chromosome. Mutations on this gene are usually rare in the early stages of the tumor (Newmark et al., 1992), while they become much more frequent in advanced and a mutation in AR gene was found in 10-20% of patients with androgen-independent cancer *(Taplin et al., 1995)*.

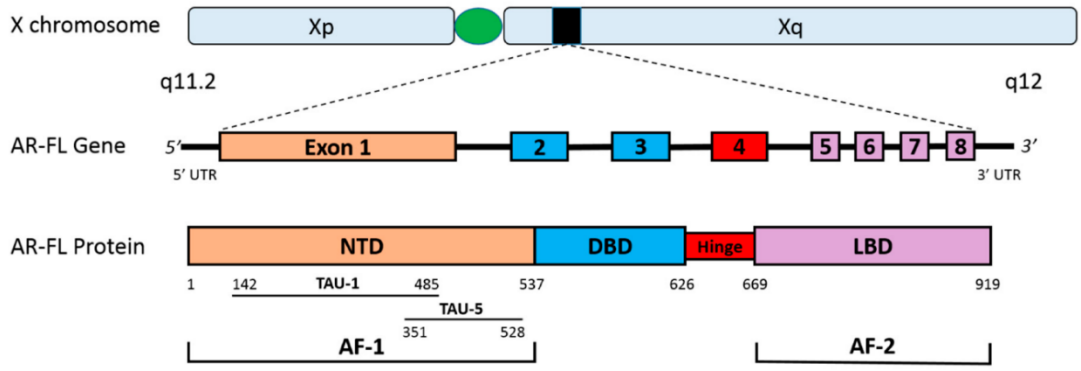


Figure 5. The human androgen receptor gene and protein. This figure depicts the gene and protein structures for the AR-FL. The AR is located on the X chromosome (Xq11.2) and is comprised of eight exons. AR-FL contains the NTD (encoded by exon 1), the DBD (encoded by exons 2–3), the hinge region (encoded by exon 4) and the LBD (encoded by exons 5–8). The strong transcriptional activity in the NTD can be attributed to the AF-1, while the LBD contains the weaker AF-2. Two major transactivation units are present in the AF-1: TAU-1 and TAU-5. Abbreviations: AF-1, activation function 1; AF-2, activation function 2; AR-FL, androgen receptor full length; DBD, DNA-binding domain; LBD, ligand-binding domain; NTD, N-terminal transactivation domain; TAU-1, transactivation unit 1; TAU-5, transactivation unit 5; UTR, untranslated region.

c) Changes in the expression of AR receptor regulating proteins: AR interacts with many core-regulatory proteins that can stimulate (co-activators) or decrease their transactivation (co-repressors), with resulting in an alteration of the transcription process. To date, 170 coregulatory proteins have been identified *(Heemers et al., 2007)*. Alterations in the balance of these proteins could promote the growth of prostate cancer cells. Co-activators, such as TIF2 and SRC1, can recruit other transcription factors to begin the process of transactivating AR-regulated genes *(Lemon et al., 2000)*. It has been shown that the concentration of TIF2 and SRC1 increases in androgen-independent phenotype expressing increased levels of AR *(Gregory et al., 2001)*. Co-repressors can form complexes with AR by inhibiting transcription of AR-regulated genes. Examples of co-repressors are NCoR and SMRT *(Liao et al., 2003)*; they can recruit histone-deacetylase, which promote the compaction of chromatin, reducing transcriptional activity.

d) Alternative activation routes: steroid hormone receptors, including AR, can also be activated by ligand-independent mechanisms. It is established that, in the presence of low concentrations of androgens and in the absence of ligands, AR can be stimulated by growth factors (IGF-I, EGF), pro-inflammatory cytokines (IL-6), forskolin and bombesin (HER2/neu) *(Saraon et al., 2011)*.

Treatments

Therapeutic treatments are based on different criteria: patient age, health status and life expectancy, stage and aggressiveness of the tumor and PSA levels. About 40% of PCa patients, including those with metastatic or locally advanced disease, die from causes unrelated to the disease, so it is appropriate to evaluate the type of therapeutic approach on the individual patient *(Albertsen et al., 1998)*. For patients with a life expectancy of less than ten years, due to an advanced age or by comorbidity, a policy of "vigilant expectation" is carried out, which provides for a postponed therapy, mostly palliative, at the time of the possible appearance of symptoms. Whereas for patients with inexpressive and early-stage cancer, the "active surveillance" is carried out, which consists of periodic and continuous monitoring of the individual in order to detect the progression of the disease in a opportune manner and thus proceed to the choice of the most appropriate treatment. Finally, treatments with anti-androgens or radical therapies are used in subjects with metastatic cancer spread. To date, there are several therapeutic strategies for the treatment of PCa:

(i) Radiotherapy → a radical treatment that uses ionizing radiation with high-energy pointed to cancer cells. It can also be considered in the postoperative stage to defend against relapse, or as a palliative cure to decrease symptoms from bone metastases. There are two most frequent techniques: external beams radiation therapy, well tolerated, such as Intensity-modulated radiation therapy (IMRT) or proton therapy; and brachytherapy or internal radiation therapy, which involves radioactive implants to insert directly into the prostate. However, side effects can occur in acute, such as cystitis and acute proctitis, and late ones that tend to become permanent.

(ii) Surgery → it includes radical prostatectomy that is applied for those tumors that are not responding to radiotherapy. It is an intervention of total removal of the prostate and seminal vesicles with the purpose of eradicate the organ confined with the mass. Generally, the lymph nodes in the pelvis can also be removed to check for metastases and possibly assess the need for further therapies. It can be performed with different techniques: open-air, laparoscopic, or using a surgical robot. However, there may be post operatory complications such as erectile dysfunction and urinary incontinence. Other types of surgical intervention are robotic or

laparoscopic prostatectomy, less invasive than a radical prostatectomy and may shorten recovery time; and bilateral orchiectomy, the surgical removal of both testicles;

(iii) Focal therapies → are less-invasive treatments that destroy small prostate tumors without treating the rest of the prostate gland. These treatments use heat, cold, and other methods to treat cancer, mostly for low-risk or intermediate-risk prostate cancer. Focal therapies are being studied in clinical trials. Most have not been approved as standard treatment options.

(iv) Hormone therapy → since PCa is an androgen-dependent tumor, the ability to reduce testosterone and DHT levels are an excellent therapeutic strategy, which has allowed it to overcome the obsolete bilateral orchiectomy (removal of the testicles, leading testosterone manufacturers). It involves administering drugs such as analogues or antagonists of the hormone releasing the hormone luteinizing (LHRH), or antiandrogens, which compete with DHT for binding with the androgen receptor. Numerous are the side effects, such as hot flashes, impotence, declining libido and others. In addition, the disease can develop drug- resistance by evolving to a form of androgen-independent phenotype. CRPC is a very aggressive tumor with very few effective therapies available for treatment, which results in a high mortality rate *(Ricci et al., 2014)*.

(v) Chemotherapy → Chemotherapy is the use of drugs to destroy cancer cells, usually by keeping the cancer cells from growing, dividing, and making more cells. Chemotherapy may help those with advanced or castration-resistant prostate cancer and those with newly diagnosed or hormone-sensitive metastatic prostate cancer. Different studies demonstrated the effectiveness of docetaxel in CRPC patients *(Petrylak et al., 2004; Tannock et al., 2004)*. There are several standard drugs used for prostate cancer. In general, standard chemotherapy begins with docetaxel combined with prednisone. Cabazitaxel is approved to treat metastatic castration-resistant prostate cancer that has been previously treated with docetaxel. It is a microtubule inhibitor. Recent research shows adding docetaxel chemotherapy to testosterone suppression therapy in those with newly diagnosed or hormone-sensitive metastatic prostate cancer significantly helps people live longer and stops the disease from growing and spreading. This chemotherapy drug

allows to reduce the size of the tumor, reduce the symptoms and pain caused by bone metastases and preserve a moderate quality of life, however, being a highly toxic substance, it is recommended to delay its intake as much as possible *(Graff et al., 2015)*.

(vi) Immunotherapy → also called biologic therapy, is designed to boost the body's natural defenses to fight the cancer. It uses materials made either by the body or in a laboratory to improve, target, or restore immune system function. For some men with castration-resistant metastatic prostate cancer who have no or very few cancer symptoms and generally have not had chemotherapy, vaccine therapy with sipuleucel-T may be an option. Different types of immunotherapy can cause different side effects.

Prostate cancer and microenvironment

The microenvironment is composed of complex connections that include stromal multipotent cells and mesenchymal stem cells, fibroblasts, blood vessels, precursors endothelial cell and secretion factors, such as cytokines *(Kenny et al., 2007)*. Tumor and host cells co-evolve dynamically through direct and indirect interaction that cause effects at molecular and cellular levels, including proliferation, metabolism, and cell growth, as well as angiogenesis, hypoxia and response adaptive and innate immune system *(Shiao et al., 2011)*.

Specific oncogenic mechanisms are related to dramatic changes in the tumor microenvironment, which, once mutated, becomes cause and consequence of the genesis of the tumor and therefore contribute to its initiation and its progression.

The manipulation of the microenvironment can be used as an approach to prevention and treatment of cancer *(Casey et al., 2015)*, targets of particular interest are the synthesis of cholesterol and its metabolites, oxidative stress and inflammatory processes *(Casey et al., 2015)* (Figure 6).

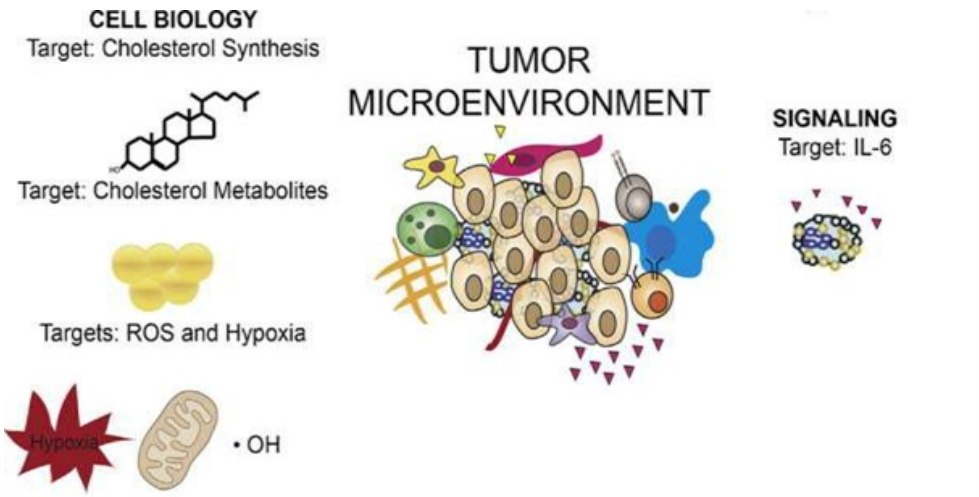


Figure 6. Tumor microenvironment and possible target involved.

Sources of cholesterol

Cholesterol and its metabolites are important for the structure and fluidity of membranes and are precursors of hormones (such as testosterone, progesterone, cortisol and estradiol). Under homeostasis conditions, cholesterol levels are regulated by the balance between cellular synthesis, diet, and elimination *(Simons et al., 2000)*. Cholesterol homeostasis is regulated by two main transcription factors: SREBP2, which increases cholesterol synthesis and uptake, and LXR, which reduces them. This balance guarantees homeostatic control. Recent evidence suggests that disorders of the homeostasis determine the accumulation of cholesterol observed in PCa.

Cholesterol metabolism plays an important role in the pathogenesis of many diseases, including prostate cancer, by regulating signal pathways inflammation and immune response *(Casey et al., 2015)*. Cancer cells have an alteration in cholesterol homeostasis, with an increase in absorption and biosynthesis and a decrease in efflux, leading to multiple effects on tumor growth, apoptosis and sensitivity or resistance to external agents *(Pelton et al., 2012)*. The metabolism of cancer cells is higher than normal cells, since it must support tumor proliferation, migration, and metastatic activities *(Warburg., 1956)*. The increase in biosynthesis, uptake and the decrease in the efflux of cholesterol from the cells are considered characteristics associated with the carcinogenesis. Cholesterol is synthesized via the mevalonate pathway, an enzymatic cascade mainly controlled by the SREBP family of transcription factors *(Horton et al., 2002)*. It is synthesized from an acetyl-CoA molecule and acetoacetyl-CoA molecule under the control of an enzymatic reaction

mediated by HMG-CoA reductase (beta-hydroxide-beta-methylglutarilCoA reductase) *(Nelson et al., 2009)*. In the tumor the activity of the enzyme HMG-CoA reductase is increased *(Caruso et al., 2002)* with an increase loss of by-product inhibition. Moreover, there is an increased cholesterol plasma lipoprotein trough LDLR and receptor SR-BI. On the other hand, any excess of cholesterol needs to be eliminated from cells to prevent cell damage. ATP-binding cassette (ABC) transporters mediate reverse cholesterol transport, a process by which excess cholesterol from peripheral tissues is returned to the liver by high-density lipoproteins. The ABCA1 transporter promotes cholesterol efflux to lipid-poor apoA-I lipoprotein leading to HDL formation (*Bovenga et al., 2015)*. ABCG1 cooperates with ABCA1 in macrophages, further maturing the HDL *(Phillips., 2014)*. Overall, these transporters together and the inhibition of the enzymes that control cholesterol synthesis and the inhibition of receptors involved in cholesterol uptake, maintain the cholesterol homeostatic state. So, inhibit cholesterol synthesis and change the balance between uptake and efflux of cholesterol could be an approach to stop the progression of the tumor.
Cholesterol is a fundamental structural component of cell membranes. It makes up about 30% of the lipid bilayer on average. Acting as essential building blocks of the plasma membranes, cholesterol plays pivotal roles in maintaining the structural integrity and regulating the fluidity of cell membranes *(Chapman., 1975)*, therefore contributing to the contribution of various membrane proteins on the cell surface. Furthermore, regarding its cell membrane-associated functions, cholesterol is also implicated in the modulation of cellular signal transmission and intracellular trafficking through contributing to lipid raft assembly and assisting the formation of endocytic pits *(Ohvo-Rekilä et al., 2002)*. Cancer cells have a high capacity to proliferate and therefore have a greater need for cholesterol than normal cells.
Cholesterol is the natural precursor of steroid hormones and its levels have been shown to affect the progression of prostate cancer. The synthesis of androgen steroid hormones can be done by low concentrations of androgens from DHEA, but also through the synthesis of cholesterol *(Hamid et al., 2013; Mostaghel et al., 2012)*. The enzymes involved in the synthesis of androgens, such as cytochrome P (CYP) 11, CYP17A, and the 3-βhydroxysteroid dehydrogenase (3βHSD), have been identified in LNCaP tumors on mice fed on a hypercholesterolemic diet. High expression of CYP17A in tumor tissues is positively correlated to high levels of plasma cholesterol *(Mostaghel et al., 2012)*. In samples of

patients with CRPC there was a significant increase in the expression of CYP17A1, 3βHSD1 e 3βHSD2 genes *(Montgomeri et al., 2008)*. Subsequently, the precursors of androgens produced by the steroidogenesis de novo must be converted to active androgens. In prostate cancers the mechanism or way to convert these precursors is under investigation. Cholesterol is involved in the *de novo* synthesis of some hormones *(Mostaghel et al., 2012)* and especially testosterone (T) and dihydrotestosterone (DHT). This pathway of hormonal production becomes particularly important in patients with CRPC because it is responsible for maintaining the constant T and DHT levels at intraprostatic level even after androgenic deprivation (ADT) therapy. T and DHT are the main androgens involved in cell differentiation and prostate homeostasis *(Van der Sluis et al., 2012)*. *In vivo* and *in vitro* data show how this share of intraprostatic T and DHT is enough to stimulate gene expression regulated by androgens and support the growth and survival of cancer cells mediated by androgen receptor (AR) *(Marks et al., 2008)*. In the circle remain some precursors of cholesterol-derived androgens such as DHEA available for synthesis. Many studies have shown that in CRPC tumors, T and DHT can be synthesized through classic or alternative pathways *(Auchus et al., 2012)*.

In addition to cholesterol, also its metabolites are associated with the progression of several tumors through the regulation and activation of oncogenes *(McDonnell et al., 2014; Lin et al., 2013)*. The enzymes in the P450 family metabolize cholesterol by producing steroids and oxysterols. Steroids, such as estrogen and androgens, are involved in the progression of cancer *(Fox et al., 2009)*; another actor involved in the progression of cancer are the oxysterols that play an important role in intracellular cholesterol homeostasis. Oxysterols are generally present in low concentrations compared to cholesterol *(Gomaraschi., 2019).* These metabolites inhibit the synthesis of cholesterol and increase its efflux through the activation of LXR *(Dufour et al., 2012; York et al., 2013)*. Liver X receptor, LXRα and LXRβ, are ligand-activated transcription factors belonging to the nuclear receptor superfamily *(Repa et al., 2000)*. They regulate cholesterol and fatty acid metabolism. The activation of LXRα and β receptors is mediated by cholesterol precursors, such as desmosterol and by oxysterols. Many of the oxysterols (e.g. 7α-, 7β-, 25-hydroxycholesterol) have anti-proliferative effects in many types of cancer *(Lin et al., 2013)*. Instead, it was recently observed that the 27-hydroxycolesterol (27HC) acts as an estrogen receptor agonist in breast cancer, and that is can also promote tumor growth and

metastasization *(McDonnell et al., 2014)*. Another study support that these metabolites could be involved in cancerogenesis and tumor progression, in fact 27-hydroxycholesterol for example could contribute to tumor growth, also in prostate cancer *(Raza et al., 2017)*. Some evidence suggests that oxysterols play a role in malignancies such as breast, prostate, colon, and bile duct cancer *(Kloudova et al., 2017)*.

Several studies have the aim to find a drug therapy useful for controlling tumor development, which targets the synthesis, transport, and homeostasis of cholesterol metabolites.

Reactive oxygen species

Reactive oxygen species (ROS), which are hydroxyl, peroxide, and superoxide, are radical and non-radical oxidant agents, which can be easily converted to one or more electrons *(Apel et al., 2004)*. They are normally generated during the metabolic processes of cells and in various enzymatic reactions, such as the electron transport chain in the mitochondria, gene expression, signal transduction and the activation of transcription factors *(Halliwell et al., 1991)*. Excessive production can lead to tissue damage and contribute to the genesis of the tumor, as it can induce tissue damage mutations and altering the microenvironment. In fact, levels of ROS in cancer cells are higher than non-tumor cells *(Ames et al., 1983)* and the alteration of proliferation, apoptosis and cancerous cells could be the result of the activation of cancer response to increased oxidative stress. Several epidemiological studies have proven the role of oxidative stress in development and progression of prostate cancer *(Khandrika et al., 2009)*, in fact many factors that are associated with the risk of this disease such as aging, alteration of androgens, loss of balance of antioxidant mechanisms, high-fat diet and pre-poor conditions are closely related to it. The balance between oxidants and antioxidants is increased radical species *(Khandrika et al., 2009)*. Many study have been carried out on the use of antioxidants, as vitamin E, in the diet in order to reduce the risk of prostate cancer, *(Khandrika et al., 2009)*; in fact in smokers, the intake of about 50 mg of vitamin E per day reported data with reduction of incidence of cancer and its statistically significant mortality compared to the placebo *(Heinonen et al., 1998)*. Failure to influence the disease of a diet rich in antioxidants suggests that ROS-neutralizing agents are not as useful as agents that inhibit their overproduction. The production of ROS, rather than accumulation, plays an important role in the evolution of prostate cancer and therefore

the use of antioxidants that only neutralize the radicals accumulated within cells doesn't have a great benefit *(Kumar et al., 2008)*. It is needed to reduce its production.

Androgens are considered fundamental to the regulation of the ROS balance in the reducing oxidative stress by inhibiting NADPH oxidase (Nox) expression and thus restoring the balance between antioxidants and oxidants. NADPH oxidase is responsible for generating ROS in the cell in form of anions superoxide undergoing dismutation forming peroxide (H_2O_2) *(Clark et al., 1999)*. These oxidations are able to play an important role in various signal events such as growth, survival and cell death becoming so critical, not only for ROS production, but also in maintaining the malignant phenotype of the tumor *(Lim et al., 2005)*. Some phenotypes of the Nox family are present in cancer cells and are absent in normal cell lines, in particular the isoforms 1 and 5. Nox1 triggers a signal pathway that induces neoangiogenesis and conversion of the cancer from quiescent to aggressive growth *(Block et al., 2006)*; the ectopic expression of Nox1 in prostate cancer cells stimulates growth, cancer and neoangiogenesis; different inhibition of the Nox5 causes growth and induction of apoptosis *(Brar et al., 2003)*. The connection between ROS production from the Nox system and the carcinogenic potential suggests that this pathway could play a critical role in tumor modulation and thus be a possible target for therapeutic intervention.

Another component involved in the redox balance in cells is oxide-reductive system of glutathione: somatic mutations that cause inactivation of GSTP1 (the coding gene for glutathione S-transferase) have been identified in the most cases of prostate cancer *(Nelson et al., 2004)*. DNA mutations at mitochondrial level are very frequent and, accompanied by impaired metabolism and various mitochondrial dysfunctions, can contribute to the pathogenesis and metastasization of the tumor *(Dakubo et al., 2006)*. It was also shown that there is a gradual increase of mutations in late prostate cancers compared to early ones, this suggests that oxidative stress causes an increase in these mutations that gradually promote mutagenesis and angiogenesis of the tumor.

Physiologically, oxidative stress grows with age for increased ROS production due to defective oxidative phosphorylation and electronic transport *(Khandrika et al., 2009)*. Mitochondrial DNA mutations/deletions do not act so only as a marker of aging, but they can also explain the increase in incidence of prostate cancer as you get older. The body opposes the development of the tumor through the activation of oncosuppressor. For example, the oncosuppressor p53 leads to apoptosis and induces hypoxia *(Celotti., 2006)*;

however, the tumor reacts to oxygen deficiency situation with two mechanisms: neoangiogenesis and activation of HIF-1 (hypoxia inducible factor). The mechanism of neoangiogenesis development of a new vascularization permit to the cells to have an adequate nutritional and oxygen intake *(Celotti., 2006)*. Activating and stabilizing HIF-1 increases the genes involved in cell survival in low oxygen conditions.

Severe pathological conditions such as inflammation and infection are associated with stress inflammation leads to increased production of ROS, which promotes the development of an inflammatory proliferation atrophy that generally cause intraepithelial prostate cancer and progressively prostate cancer. Chronic prostate inflammation, as prostatitis, leads in most cases to damage to stromal and epithelial cells; inflammatory cells, including macrophages, which are attracted to the site of inflammation, can be found in secretions chronic prostatitis *(Khandrika et al., 2009)*. These cells activate non-specific immune defense and there is a rapid increase in hydroxyl radicals, superoxide and peroxide in prostate tissue. The continued exposure of the tissue at the source of inflammation can lead to a significant increase in species causing changes in protein structures and functions, genetic alterations and post-translational DNA changes *(Olinski et al., 2002)*, these changes will bring epithelial cell proliferation to compensate for tissue damage and therefore they can induce prostate cancer *(Naber et al., 2000)*. Pro-inflammatory agents accumulate in the prostate region cause inflammation and high PSA levels that could represent pre-malignant changes *(Sutcliffe et al., 2006)*; in addition to the cytokines and chemokines that increase the production of ROS, many other molecules and signal pathways activated in immune cells can lead to development of the cancer.

Inflammatory molecules

Chronic inflammation is involved in the regulation of cellular events in prostate carcinogenesis. Tumors are not epidemiologically linked to inflammation *(Nguyen et al., 2014)*; In fact, the frequency and distribution of tumors does not appear to be related to inflammation. However, tumors have numerous inflammatory infiltrations *(Nguyen et al., 2014)* who are responsible for atrophy in the prostate and predisposition to PCa. Molecular mechanisms that bind inflammation and cancer include damage to DNA, reduced immune response and alteration of the tumor microenvironment *(Nguyen et al., 2014)*. The microenvironment has alterations in the cytokine balance, chemokines, transcription

factors and ROS. Of all the cytokines, IL-6 is the most studied and more involved in the progression of prostate cancer. IL-6 is a powerful inflammatory cytokine that regulates the innate immune response, differentiation and activation of B and T lymphocytes, and thus the defence against infections, but it has recently been discovered to have an important role on the microenvironment *(Casey et al., 2015)*. High plasma levels of IL-6 are associated with some pathologies such as rheumatoid arthritis, insulin resistance, sepsis and precisely cancer *(Naugler et al., 2008)*. IL-6 interacts with its own receptor on the cell membrane (IL6Ralpha/gp80) and with a signal that transduce the glycoprotein (gp130) *(Naugler et al., 2008)*. While the first one is mainly present in immune cells *(Silver et al., 2008)* and in hepatocytes, gp130 is expressed by many types of cells, including endothelial and tumor cells. IL-6 active JAK/ STAT pathway *(Naugler et al., 2008)* and the transcription factor STAT3, which induces: cancer survival, proliferation and metastasization; neoangiogenesis and the expression of factors that suppress the immune response in microenvironment *(Chang et al., 2013)*.

IL-6 can therefore be a therapeutic target; in fact, anti-IL-6 antibodies increase the cytotoxic effect of chemotherapy drugs and reduce tumor growth, macrophagic infiltration and angiogenesis *(Coward et al., 2011)*. Plasma concentrations of IL-6 are high in patients with untreated metastatic cancer or CRPC compared to patients who have a localized form *(Adler et al., 1999)* and this particularly occurs in bone metastases where interleukin plays a key role.

LNCaP, who are constantly exposed to IL-6, develop an increased invasiveness because interleukin induces epithelial-mesenchymal transition or EMT, as determined from the expression of molecular markers, such as E-cadherin and fibronectin *(Shiota et al., 2013)*. Silencing of IL-6 results in an attenuated EMT in transgenic mice with adenocarcinoma in both *in vitro* and *in vivo* studies *(Wu et al., 2012)*. EMT is a process through which epithelial cells lose their polarity and cell-cell adhesion and they get migratory and invasive properties to become mesenchymal stem cells, these are multipotent stromal cells that can differentiate into a variety of types of cells. It has been seen that IL-6 in LNCaP is also implicated in the pathogenesis of bone metastasis as it induces differentiation of osteoblasts; in PC-3 castration-resistant cell lines, an aggressive cancer model hormone-independent, the expression of interleukin in osteoblasts increases and osteoclast genesis *(Morrissey et al., 2010)*, key events stabilization of bone metastases. IL-6 released by

prostate cancer cells in the microenvironment stimulates the activity of osteoblasts and, as a result, stimulates the growth of cancer *(Nguyen et al., 2014)*. In autopsy models is increased the expression of IL-6 in bone metastases and other metastatic tissues are less involved *(Morrissey et al., 2010)*, this indicates that it acts by promoting process on both tumors and the bone microenvironment. In addition to the growth and acquisition of metastatic potential, the pro-carcinogen effect of IL-6 is also linked to the regulation of androgens receptors AR. IL-6 undergoes a functional transition from a paracrine growth inhibitor to an autocrine promoter during cancer progression *(Chung et al., 1999)*; cell alterations in CRPC include amplification and mutation of AR, dysregulation of co-regulatory protein and growth factors that can activate AR with a ligand-independent mechanism *(Zhu et al., 2008)*. In conclusion, IL-6 can regulate the expression of androgen-responsive genes in androgen deficiency conditions and is likely to contribute to the onset of the CRPC.

Lipoproteins

Lipids, such as cholesterol and triglycerides, are insoluble in water these lipids must be transported in association with proteins in the circulation. Lipoproteins are soluble complexes of proteins and lipids that transport lipids in the circulation. Lipoproteins are complex particles that have a central hydrophobic core of non-polar lipids, primarily cholesterol esters and triglycerides. This hydrophobic core is surrounded by a hydrophilic membrane that consist in phospholipids, free cholesterol, and apolipoproteins. These aggregates deliver lipids in plasma, particularly triglycerides, free cholesterol, and esterified cholesterol (Figure 7). The different combinations of lipids and proteins generate lipoproteins with different densities: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). *(Roheim et al., 1981)*.

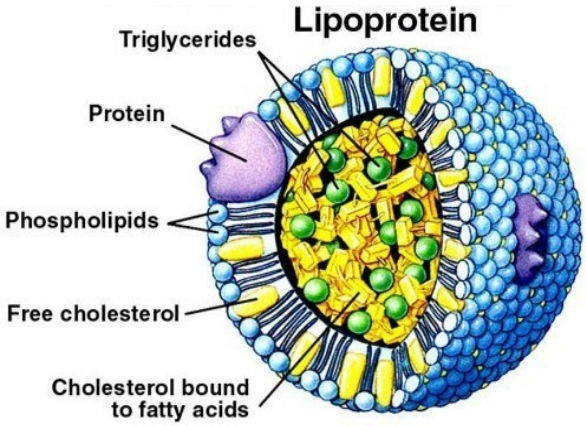


Figure 7. Structure of lipoproteins

The nomenclature and classification of serum lipoproteins were originally determined by the laboratory technique used for their separation or isolation. These included mainly electrophoretic, ultracentrifuge, and immunological techniques. Accordingly, lipoproteins have been classified based on their electrophoretic mobility, hydrated density, and apolipoprotein composition. Each class includes heterogeneous particles by composition, size, and biological function; in particular, the biological function of these classes is determined by the site where they are synthesized, the lipid composition and the content of apolipoprotein. At least ten different types of apolipoproteins were found in human plasma, distinguishable by size, specific interactions and distribution in the various classes of lipoproteins. These protein components, in addition to being structural elements of lipoproteins, also determine what are their functional properties. Moreover, they can act as ligands for specific receptors for lipoproteins (the first step of the cell uptake of lipids) and can act as cofactors for lipolytic enzymes, such as lecithin cholesterol acyltransferase (LCAT) and lipoprotein lipase (LPL). Apolipoproteins are therefore directly involved in the metabolism of lipoproteins. The starting point of the metabolism of lipids taken through the diet is their absorption at the level of the intestinal mucosa. However, lipids must be soluble in biological fluids before they are absorbed. This is done thanks to bile acids, molecules synthesized in the liver from cholesterol with tensioactive action, which allow the formation of micelles consisting of fatty acids and monoglycerides. These are obtained for hydrolysis of triglycerides taken with the diet, using intestinal lipase. These micelles will then be absorbed by enterocytes. The absorbed fatty and monoglycerides are reassembled into triglycerides which, together with cholesterol derived from diet and specific proteins,

will form the chylomicrons. These particles will be secreted by the enterocyte in the lymphatic circle and then into the bloodstream, from where most of the contained lipids will be released to the cells for use. The remains of the chylomicrons, called remnants, are picked up by the liver and they will create endogenous lipids. In this way, triglycerides and esters of cholesterol synthesized at the liver level are transferred into the endoplasmic reticulum at apoB-100 to give the VLDL. Once in circulation, the LPL releases free fatty acids from triglycerides, which will be accumulated by adipocytes. This loss of triglycerides converts VLDL into IDL which, by further removal of triglycerides, is converted to LDL, lipoproteins that release cholesterol to extrahepatic tissues and then are recalculated by the liver. HDL, lipoproteins involved in the reverse transport of cholesterol, are also crucial, in fact they pick up excess of cholesterol and transport it from the periphery to the liver, where cholesterol will be used both for its functions (synthesis of bile acids, synthesis of cell membranes), and for the synthesis of new VLDL (Figure 8).

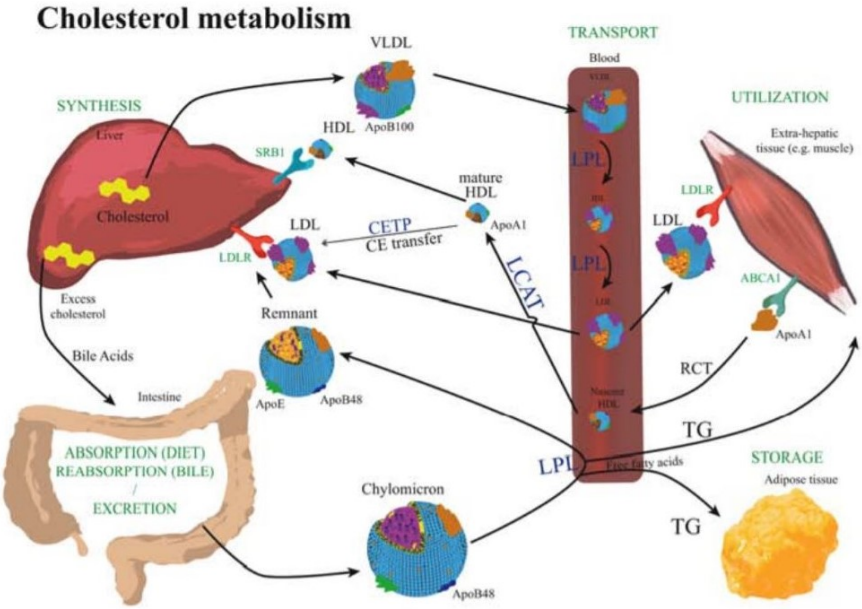


Figure 8. Cholesterol synthesized in hepatocytes is transported as VLDL to the blood circulation. The VLDL contain TG, fatty acids, free cholesterol and phospholipids. VLDL particles are transported by B100 apolipoproteins. VLDL is then remodelled and transformed into IDL and LDL by the action of LPL in the blood circulation. The cholesterol could also be transported by chylomicrons. Chylomicrons are synthesized by the enterocytes cells in the intestine tissue after diet absorption or bile acids re-absorption. The chylomicrons transport the cholesterol from intestine to the circulation via the Apolipoprotein B48. LPL triggers the transformation chylomicrons into remnants with the release of free fatty acids (FFA) in the circulation. These FFA will be stored in adipose tissue in the form of TGs or could be oxidized by non-hepatic tissues such as muscles. LDL particles and remnants are removed from the circulation through interactions with LDL receptors. The excess of cholesterol can be cleared from tissue via the reverse cholesterol transport (RCT) via the ApoA1 lipoprotein.

Low density lipoprotein

LDL (low density lipoprotein) are lipoproteins rich in cholesterol and cholesterol esters and apoB-100 is the main lipoprotein present. These particles carry about 70% of plasma cholesterol to peripheral tissues in healthy subject, such as muscle and fatty tissue, which possess receptors for LDL that recognize apoB-100. The LDL particles are isolated from human plasma by ultracentrifugation, they have a density range of 1.019-1.063 g/ml and a molecular weight from about 2 to 3.5 x 10^6 and a diameter from 200 to 250 Å *(Alaupovic et al., 1972)*.

LDL are very heterogeneous family of lipoproteins and can be classified based on their density and shape. The size of an LDL particle depends on how much lipid is in the core, and the lipid content naturally determines its density. Thus, smaller LDL is denser, larger LDL is lighter, and the two qualities are largely equivalent. Based on their density three subclasses were defined: medium-density LDL (1.030-1.039 g/ml), high-density LDL (1.039-1.063 g/ml), and low-density LDL (1.019-1.030 g/ml) *(Carter et al., 1994)*. These LDL subclasses appear to have a higher net negative charge. As a result, they bind with less affinity to the LDL receptor and their absorption and degradation by LDL-R proceeds at a slower rate than medium-density LDL *(Nigon et al., 1991)*. In addition, low-density LDL are more susceptible to oxidation and are therefore considered more pro-atherogenic LDL.

Metabolism

The levels of plasma LDL are determined by the rate of LDL production and the rate of LDL clearance, both of which are regulated by the number of LDL receptors in the liver. The production rate of LDL from VLDL is partially determined by hepatic LDL receptor activity with a high LDL receptor activity resulting in a decrease in LDL production due to an increase in IDL uptake. Conversely, low LDL receptor activity results in an increase in LDL production formation due to a decrease in IDL uptake. With regards to LDL clearance, approximately 70% of circulating LDL is cleared via hepatocyte LDL receptor mediated endocytosis with the remainder taken up by extrahepatic tissues. An increase in the number of hepatic LDL receptors therefore increases LDL clearance leading to a decrease in plasma LDL levels. Conversely, a decrease in hepatic LDL receptors slows LDL clearance leading to an increase in plasma LDL levels. Thus, the level of hepatic LDL receptors plays a key role in regulating

plasma LDL levels. LDL have a threefold destiny: most are removed from the liver by interaction with LDLR, one part is picked up by peripheral tissues always using LDLR, and the other is degraded through scavenger receptors. Cholesterol that enters in hepatocytes can be either incorporated into membranes or converted into bile acids or re-esterified by ACAT to be stored and deposited within cytosolic droplets *(Franceschini et al., 2006)*.

Plasma levels of LDL are the result of the balance between generated LDL and re-uptaked LDL; these are directly dependent on the number of their liver receptors. About 70% of circulating LDL are re-uptaked by the LDL receptor, located on the hepatocyte membrane, which recognizes apoB-100 by internalizing the lipoproteins for endocytosis. As a result of the endocytosis process, lipoproteins are degraded at the lysosomes level, releasing unesterified cholesterol and fatty acids, while the receptor can be recycled. The amount of receptors for LDL is regulated directly from the cellular content of cholesterol: at the decrease of intracellular cholesterol levels the transcription factor sterol regulatory element binding protein (SREBP) is transported from the endoplasmic reticulum to the Golgi apparatus, where it is activated by the action of some proteases; activated SREBP is transported to the nucleus by stimulating gene transcription for the LDL receptor and other genes, including hydrossimethylglutaril-CoA reductase (HMG-CoA reductase) enzyme essential for cholesterol biosynthesis. Conversely, as intracellular levels of inactive SREBP cholesterol increase, it remains at the level of the endoplasmic reticulum, not stimulating the synthesis of LDL receptors (*Morita et al., 2003)*. In the intracellular compartment, unesterified cholesterol can undergo oxidation reactions, generating compounds capable of activating the liver X receptor (LXR) belonging to the nuclear receptor family *(Åstrand et al., 2016)*, which stimulate ubiquitination and receptor degradation for LDL. In this way the cell can monitor the need for cholesterol accordingly the activity of LDL receptors. Another factor regulating the number of LDL receptors on the cell surface is the proprotein convertase subtilisin/kexin type 9 (PCSK9) protein belonging to the convertase family. Following a self-cleavage process it is secreted in the bloodstream, going to interact with LDLR: the link between PCSK9 and LDLR inhibits the ability to recycle post-internalization *(Rallidis et al., 2016)*, directing the receptor towards lysosomal degradation.

High density lipoprotein

High density lipoproteins (HDL) are a heterogeneous family of lipoproteins divided in different subclasses according to their density, shape, size, charge and lipoprotein composition. HDL have a density between 1.063 and 1.21 g/ml and have a diameter between 70 and 130 Å and a mass between 200 and 400 kDa *(Rothblat et al., 2010).* HDL have a high protein content, they are in fact made up of 50% protein and 50% lipids, and they are the highest density lipoprotein class. Most of plasma HDL have a spherical form with a nucleus of non-polar lipids (cholesterol and triglycerides), surrounded by a monolayer of polar lipids (phospholipids and unesterified cholesterol) and apolipoproteins *(Lund-Katz et al., 2010).* Lipids are poorly soluble in water environment and are used to be organized into micelles in plasma, while apolipoproteins are more soluble as they consist of hydrophilic and hydrophobic regions *(Gu et al., 2010; Rocco et al., 2009).* The association between lipids and proteins is therefore governed by hydrophobic interactions, where the apolar chains of fatty acids and portions of proteins with non-polar amino acids are excluded from the water environment. Apolipoproteins, phospholipids and unesterified cholesterol, characterized by an "appreciable hydrophilicity", can easily change between different circulating lipoproteins or with other lipid surfaces, such as cell membranes. Non-polar lipids, on the other hand, have a low exchange potential, can be transferred to other lipoproteins thanks to the action of specific transport proteins *(Tall et al., 2000).* A lower percentage of circulating HDL are discoidal particles, organized in a double layer of polar lipids, but without hydrophobic cores. The disc is surrounded by apolipoproteins with polar amino acid residues facing the water environment, while non-polar ones facing the double layer *(Mishra et al., 1994).*

The main protein component of HDL, consisting of apolipoproteins and enzymes, is represented by 70% apoA-I, followed by 20% apoA-II, while among the smaller protein components there are several proteins and enzymes present in the various subclasses of HDL. ApoA-IV, C-peptide, apoE, lecithin cholesterol acyltransferase (LCAT), cholesterol transfer protein (CETP), phospholipid transfer protein (PLTP), paraoxonasis (PON) and platelet activation factor acetyl hydrolase (PAF-AH) are examples of proteins circulating in plasma. About 48 types of HDL-associated proteins have recently been highlighted, most

are not apolipoproteins and are a minority component of the protein fraction of HDL; it is not yet clear how they interact with the lipid fraction *(Heinecke, 2009).*

The heterogeneity of HDL is defined by different actors (Figure 9):

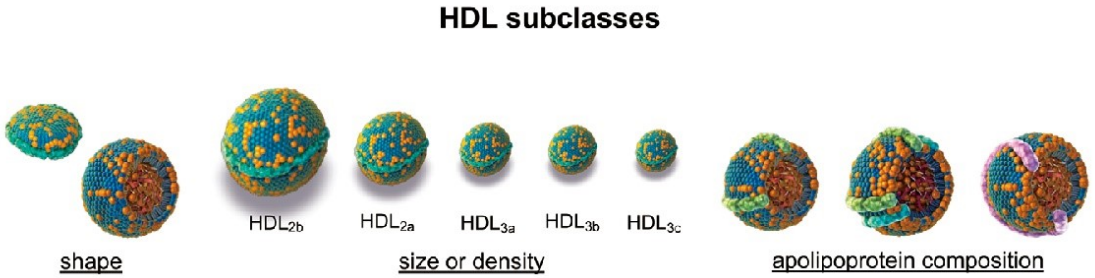


Figure 9. Structural and compositional heterogeneity of high-density lipoprotein (HDL).

a) Density

Depending on density, four different subclasses of HDL have been identified. The main subclasses are: HDL_2 and HDL_3. The first are large, rich in lipids and sediments in the region between 1.063 and 1.125 g/mL, while the other ones are smaller, rich in proteins and sediments in the region between 1.125 and 1.21 g/mL *(Schaefer et al., 1979)*. These two subclasses differ not only in density, but also in diameter and lipids-protein composition, with an inverse correlation between density and size. Two other minor subclasses are HDL_1 and HDL_4, which have been identified categories of subjects. HDL_1 are larger and less dense than HDL_2, are characterized by a greater total cholesterol and apoE content and have been identified in subjects with high levels of HDL-C, such as the genetic deficiency of CETP *(Weisgraber et al., 1980)*. HDL_4 are smaller and denser than HDL_3, contain mainly apoA-I, are spherical in shape and have been found in patients with aβlipoproteinemia or with high levels of triglycerides *(Deckelbaum et al., 1982)*.

b) Shape and surface charge

Lipoproteins are separated by electrophoresis on agarose gel based on their surface charge, depending on the charge they will have a certain electrophoretic mobility. They can occupy different positions: β, pre-β and α. Mature, spherical HDL migrate to position α, discoidal or nascent HDL in the pre-β. The α HDL are the majority of circulating HDL, they have a high surface charge compared to their mass, since the relative content of polar proteins and lipids (phospholipids, sphingolipids) that characterizes them is high *(Rothblat et al., 2010).*

The pre-β HDL, on the other hand, are discoidal in shape and have a different lipid and protein composition *(Kunitake et al., 1987)*.

c) Particle size

Using the polyacrylamide gradient electrophoresis technique (GGE), 5 distinct HDL subclasses based on their size are identified: HDL_{3c}, 7.2-7.8 nm; HDL_{3b}, 7.8-8.2 nm; HDL_{3a}, 8.2-8.8 nm; HDL_{2a}, 8.8-9.7 nm; HDL_{2b}, 9.7-12.0 nm *(Nicholls et al., 1986)*.

d) Apolipoprotein composition

HDL can be divided into two major subclasses: particles containing only apoA-I, called LpA-I, and particles containing also apoA-II, called LpA-I:II *(Cheung et al., 1984)*. ApoA-I is produced by the liver and a minor part derives from the intestine; it is secreted in a form poor in lipids *(Dixon et al., 1992)* and later converted to pre-β HDL by acquiring cholesterol and phospholipids through interaction with the transporter ATP-binding A1 cassettes (ABCA1). Cholesterol in the pre-β HDL is converted thanks to the action of LCAT resulting in the conversion of the LpA-I-pre-β HDL in LpA-I-α HDL. Also the apoA-II is produced by the liver and intestine and is associated with LpA-I to form LpA-I:II *(Rosenson RS et al., 2008).* The LpA-I:II particles always migrate to the alpha position *(Cheung et al., 1984)*, suggesting that this subclass is composed only by spherical particles. The role of apoA-II in HDL metabolism and a possible difference between LpA-I and LpA-I:II as risk factors in cardiovascular disease have not been clarified yet, although the presence of apoA-II appears to reduce cholesterol capture by liver scavenger receptors type BI (SR-BI) *(De Beer MC et al., 2001).*

Metabolism

HDL metabolism is a complex process that involve synthesis and secretion of the main protein components of HDL, followed by the acquisition of the lipid component and the assembly and generation of mature particles. HDL components are catabolised independently from each other and frequently exchanged between different lipoproteins. Apolipoproteins A-I and A-II are secreted as components of lipoproteins rich in triglycerides or very low-density lipoproteins (VLDL) released by the liver and chylomicrons released by the small intestine *(Ikewaki et al., 1993).* In plasma, lipoprotein lipase (LPL) permit the lipolysis of these lipoproteins, with dissociation of surface components such phospholipids, cholesterol and apolipoproteins. These circulating components can organize to form

nascent HDL or incorporate into HDL already present in the circle thanks to the action of transfer proteins, such as PLTP *(Huuskonen et al., 2001).* Nascent HDL can also be formed, through a mechanism that involves the secretion of apoA-I by the liver and intestine, in a lipid-poor form *(Chisholm et al., 2002; Kiss et al., 2003).* Thanks to the interaction with ABCA1, the apoA-I can acquires cholesterol and phospholipids from hepatocytes and enterocytes, leading to the formation of pre-β HDL *(Brunham et al., 2006; Timmins et al. 2005)*. Once in circulation, nascent HDL are enriched with unesterified cholesterol from VLDL or other peripheral tissues *(Movva et al., 2008).* Cholesterol is esterified by LCAT which, through a transesterification reaction *(Jauhiainen et al., 1988)*, catalyzes the transfer of a molecule of fatty acid from lecithin (phospholipid) to free cholesterol with formation of lysolecithin and esterified cholesterol. This reaction uses as an enzyme cofactor the apoA-I and the esterified cholesterol, that has a lipophile nature, migrates to the inner core of the nascent HDL. The migration to the core allows to maintain a concentration gradient that permit further transfer of unesterified cholesterol from peripheral cells and other lipoproteins to HDL and leads to the formation of mature spherical HDL (HDL_3) *(Calabresi et al., 2010)*.

HDL_3 can (i) interact in plasma with CETP, which transfers triglycerides from lipoproteins containing apoB (VLDL/LDL) in exchange for esterified cholesterol from HDL; HDL deplete from esterified cholesterol, increase in size and decrease in density for enrichment in triglycerides, turning into HDL_2 *(Zechner et al., 1987).* The triglycerides and phospholipids of HDL_2 are then hydrolyzed by lipolytic enzymes resulting in the dissociation of free apoA-I, which is in circulation and is eliminated by kidney (*Jahangiri et al., 2005)*, and the conversion of HDL from spherical to discoidal *(Clay et al., 1992; Rye et al., 2004)*. (ii) be recognized by the scavenger receptor type BI (SR-BI), which mediate the uptake from liver of esterified and unesterified cholesterol by HDL without the internalization or degradation of lipoprotein itself *(Rigotti et al., 1997),* thus generating HDL poor in cholesterol that will be incorporated into other circulating HDL *(Webb et al., 2004)* or eliminated through the kidney *(Wang et al., 1998).*

HDL can be removed from circulation by endocytosis mediated by receptors, such as SR-BI *(Eckhardt et al., 2004)* or as the ATP-synthase chain β *(Martinez et al., 2003),* which involves the degradation of the entire particle. In most cases, however, the lipid and protein portions of HDL are metabolized separately and with different mechanisms; this involves

dissociating apolipoprotein A-I from lipids, that can interact with SR-BI *(Rigotti et al., 1997),* be transferred via CETP *(Zechner et al., 1987)* and PLTP *(Jauhiainen et al., 1993)* to other lipoproteins, or be hydrolyzed by lipolytic enzymes *(Jahangiri et al., 2005).* Less well known is the clearance of the protein fraction. Different studies have identified the kidney as the main site of degradation of the unbound or poor in lipids apoA-I, since this can be easily removed from circulation through glomerular filtration. Once filtered, the apoA-I is completely absorbed at the level of the proximal convoluted tubule and is therefore not detectable in urine *(Moestrup et al., 2005).* A membrane receptor expressed in the proximal convoluted tubule, cubilin, binds the apoA-I with great affinity and mediate its endocytosis *(Kozyraki et al, 1999; Moestrup et al., 2000)* and, considering that all ligands of this receptor are destined for lysosomal degradation after internalization, it is probably that apoA-I will take the same fate. Human studies on metabolic turnover have shown that apoA-I has a catabolism rate that is significantly higher than in apoA-II and that its form not associated with lipids is generally catabolized faster than that found in HDL *(Schaefer et al., 1982).* As has been highlighted by several studies, the kidney is also able to filter HDL particles in a way dependent on their charge and size characteristics. For example, it has been observed that small, positively charged and disco-shaped HDL such as preβ-HDL cross the glomerular barrier more easily than mature HDL *(Braschi et al., 1999).*

HDL and cardiovascular risk

Atherogenesis is a multiphase process characterized by the accumulation and subsequent modification, such as aggregation, oxidation and/or glycosylation, of low density lipoprotein (LDL) in the intimae of the arterial wall. Oxidized LDL cause chronic damage to the endothelium that expresses cell adhesion molecules (CAMs) and releases pro-inflammatory cytokines, which promote the adhesion and migration of circulating monocytes within the intimae. The recruited monocytes are activated and differ in macrophages which, through the phagocytosis of lipoproteins, turn into foam cells characteristic of the lipid streaks. The foam cells produce cytokines and growth factors that induce the migration of smooth muscle cells from the medium to the intimae; it proliferate, differentiate and synthesize extracellular matrix, resulting in the transformation of the lipid streaks in advanced lesions *(Lewis et al., 2011)*. The progressive damage of vascular endothelium and the formation of the atherosclerotic plaque increase the risk of

cardiovascular disease (CVD). Cardiovascular diseases are still among the main causes of mortality and mobility in industrialized countries *(Lopez et al., 2006).* Many epidemiological studies have shown that low levels of HDL and apoA-I cholesterol were associated with an increased incidence of CVD, suggesting that by focusing on the increase in HDL it was possible to reduce the incidence of cardiovascular events *(Castelli et al., 1986; Gordon et al., 1977).*

HDL purified from human plasma are unsuitable for drug development, because of their heterogeneity, problems related to large-scale production and safety concerns. The infusion of synthetic HDL (sHDL) is a possible therapeutic approach to increase HDL circulating pool *(Calabresi et al., 2006)*. Synthetic HDL are discoidal particles containing phospholipids, mainly phosphatidylcholine, and apoA-I or its variants, which reproduce the structure of the nascent HDL *(Calabresi et al., 2006)*. The apolipoproteins may be isolated from human plasma, through a combination of ultracentrifugation techniques and chromatographic separations, or by fractional precipitation in ethanol *(Brewer et al., 1978)*. However, these procedures do not allow the obtaining of large amounts of protein required for sHDL production. An innovative approach involves the recombinant DNA technology, which allows to obtain even site-specific mutants, with better functional characteristics than the wild-type protein *(Sirtori et al., 1999)*. Even the apoA-I mimetic peptides (peptides designed to mimic the ability of apoA-I to form amphipathic helices) can be used in combination with phospholipids to produce sHDL, with the advantage of being able to be produced by synthesis on a large scale *(Garber et al., 2001)*. Phosphatidylcholine (PC) is the most abundant phospholipid in plasma HDL and therefore the best candidate as lipid component in sHDL production. sHDL have the same endogenous HDL metabolism: after infusion, sHDL acquire unesterified cholesterol from VLDL or other peripheral tissues and become LCAT substrate. The esterification of cholesterol leads to conversion of the sHDL disk-shaped into spherical particles, like the mature HDL of endogenous derivation. Even the plasma clearance of sHDL and endogenous particles is similar, the lipid and the protein portions are independently catabolised each other (Raut et al., 2018). To be effective as therapeutic agents in cardiovascular diseases, sHDL must be able to reproduce atheroprotective activities of plasma HDL. *In vitro* and *in vivo* studies showed the ability of sHDL to participate in all the main processes of RCT. They interact with ABCA1 transporters, ABCG1 and SR-BI, promoting cholesterol efflux from peripheral cells. sHDL also maintain

the antioxidant properties, stimulate the production of nitric oxide and retain the action of endothelial protection of endogenous HDL *(Calabresi et al., 2006)*.

Reverse Cholesterol Transport

The main mechanism through HDL perform the function of atheroprotection is the reverse transport of cholesterol (RCT) *(Rader et al., 2006; Ross et al., 1973),* a process by which cholesterol is transmitted from peripheral tissues (including the arterial wall) to the liver to be eliminated through bile. In addition, HDL have other activities that can contribute to the anti-atherogenic effect, such as maintaining endothelial function and antioxidant effect. The atheroprotection process is HDL dependent *(Calabresi et al, 2010)*.

The main function of HDL is to promote the removal of cholesterol from peripheral cells, including macrophages at the level of the arterial wall, and to transport it to the liver for elimination. This process is called reverse cholesterol transport and is composed of various stages (Figure 10).

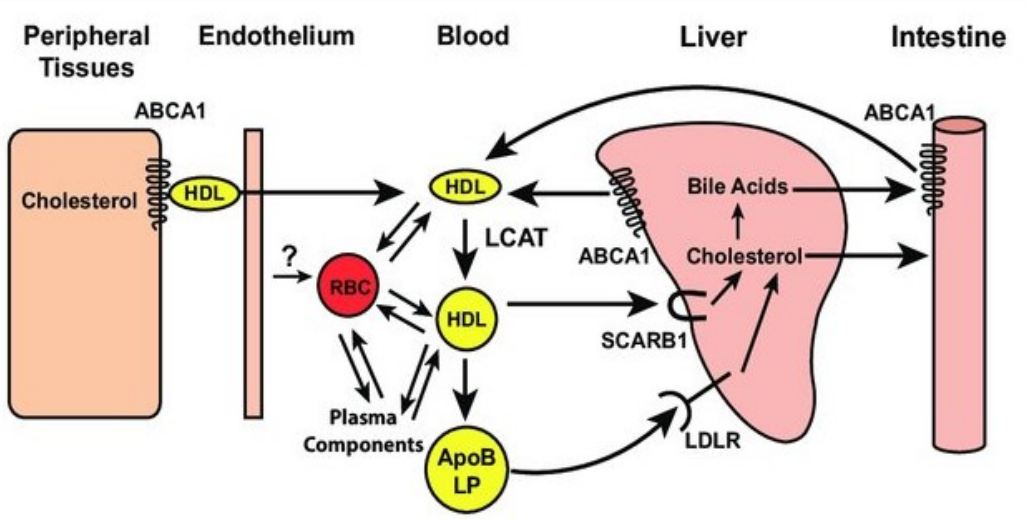


Figure 10. Schematic of reverse cholesterol transport in the blood. Free cholesterol in peripheral tissues is effluxed by ABCA1 transporters to highdensity lipoprotein (HDL), and transported into the vascular space. Lecithin cholesterol acyl transferase (LCAT), present on HDL, esterifies free cholesterol. The cholesterol esters formed by the LCAT reaction are either taken up by SCARB1 receptors on hepatocytes or transferred to apolipoprotein B containing lipoproteins (ApoB LP) and taken up into hepatocytes by the low density lipoprotein receptor (LDLR). Red blood cells (RBCs) may also participate in reverse cholesterol transport by accepting cholesterol from vascular endothelial cells and participating in a cholesterol exchange with lipoproteins and plasma components, such as albumin.

The first phase of the RCT involves the efflux of unesterified cholesterol by peripheral cells to extracellular acceptor, such as HDL or non-lipidated apoA-I and is considered the limiting step the entire process. Cholesterol efflux can occur by passive diffusion according to

concentration gradient, or by active transport *(Jessup et al., 2006).* Simple diffusion is a non-saturated and ATP-independent process, which mediate the two-way flow of cholesterol between cell membranes and acceptors such as lipoproteins. Only cholesterol located on the extracellular side of the membranes (about 3-5% of the total) can move passively *(Raggers et al., 2001)* and therefore most important ways are mediated by transport proteins. These pathways have as cholesterol acceptors different subclasses of HDL and include: one-way active transport (cell to acceptor) ATP-dependent mediated by the ABCA1 transporter, ATP-dependent transport mediated by the ABCG1 transporter and facilitated two-way ATP-independent diffusion mediated by the SR-BI receptor. The main acceptor of cholesterol in the way mediated by ABCA1 is the apoA-I in lipid or lipid-poor form; its interaction with ABCA1 promotes the efflux of cholesterol and phospholipids, leading to the formation of nascent HDL, which can remove additional cholesterol from cells via the same pathway *(Favari et al., 2004).* The membrane transporter ABCG1 is also involved in the process of cholesterol efflux from macrophages. In this way, both the nascent HDL, often derived from the way mediated by ABCA1, and the mature HDL, are acceptors of cholesterol *(Rothblat et al., 2010).* The pathway mediated by SR-BI prefer as acceptor mature HDL. This receptor permits the two-way transport, so it is possible that HDL rich in esterified cholesterol will function as donors *(Favari et al., 2009; Jessup et al., 2006).* Cholesterol efflux is a complex and saturable process, dependent on both the bioavailability of transporters on cell membrane and cholesterol acceptors suitable in extracellular space.

The second phase of the RCT involves the plasma conversion of HDL. The cholesterol is in fact esterified by LCAT and migrates in the lipoprotein, resulting in a progressive increase in size. Migration allows the removal of unesterified cholesterol from the lipoprotein surface and helps to maintain the concentration gradient needed for the efflux from donor cells.

The third and final stage of the RCT consists of the uptake of cholesterol by the liver and can take place through two main pathways: (i) cholesterol esters are transferred to lipoproteins containing apoB (VLDL/LDL) in exchange for triglycerides using the action of the CETP transport protein. Lipoproteins containing apoB are then catabolised by the liver for internalization mediated by their liver receptors *(Schwartz et al., 2004).* (ii) cholesterol esters contained in HDL are selectively picked up by hepatocytes for interaction with the

SR-BI receptor. At the liver level, esters of cholesterol are hydrolyzed and unesterified cholesterol are excreted in the bile *(Yu et al., 2002).*

Endothelial protection

Vascular endothelium is a structurally simple but extremely complex tissue from a functional point of view; in fact it is not only a component of the vessel wall, but is responsible for the synthesis and the release of numerous substances that regulate vascular tone, inflammation, homeostasis and the integrity of the endothelium itself *(Calabresi et al., 2003)* (Figure 11).

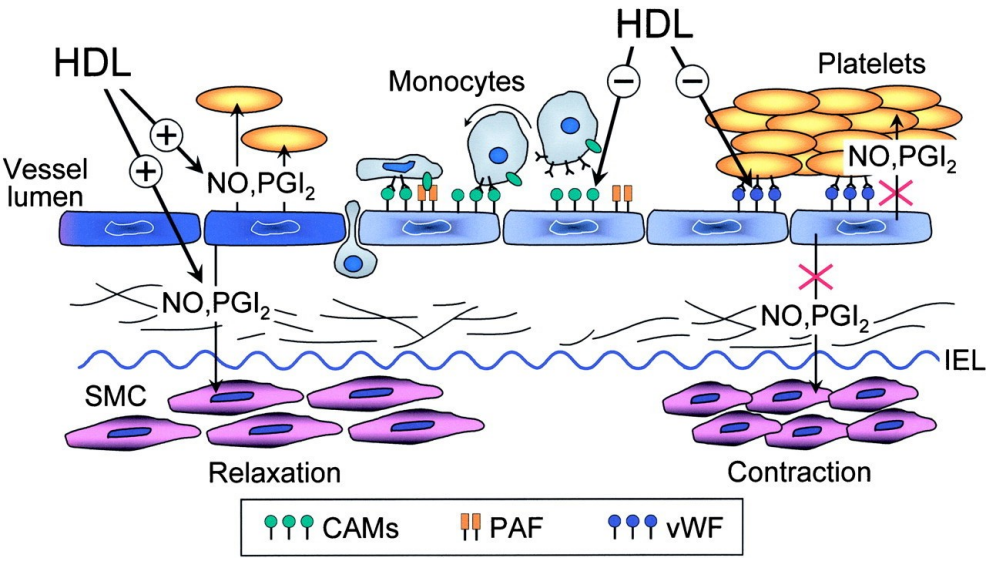


Figure 11. Multiple biological actions of HDL on vascular endothelium. Functional endothelial cells are in dark blue; dysfunctional endothelial cells are in light blue.

Endothelial cells (ECs) release vasoactive molecules, such as nitrogen monoxide (NO) and prostacyclin (PGI_2), which mediate relax of vascular tone and exert a powerful antithrombotic effect. The decrease in their bioavailability is one of the characteristics of the endothelial dysfunction, a key event in the onset and progression of the atherosclerotic lesion. The dysfunctional endothelium also expresses cell adhesion molecules (CAMs) on its surface, these have a pro-adhesive effect and contribute to atherogenesis by mediating the adhesion of circulating monocytes to the arterial wall and favour their migration in the intimae. Several *in vitro* and *in vivo* studies have shown that HDL are capable of increasing the bioavailability of NO and PGI_2 through different mechanisms and how they significantly

reduce CAMs performance, helps to preserve the integrity of the cellular monolayer and thus reduce the development of atherosclerotic diseases *(Calabresi et al, 2003)*.

Nitric oxide (NO) is a signal molecule produced by endothelium. This molecule promotes vasodilatation as it acts on smooth muscle cells by inducing their relaxation. Reducing NO bioavailability is a feature of the endothelial dysfunction. Different *in vitro* studies on endothelial cells shown the ability of HDL to increase the production of NO, increase the synthesis of eNOS and its activation *(Gomaraschi et al., 2007; Kuvin et al., 2002; Ramet et al., 2003)*. NO is produced by NO synthase (eNOS), an enzyme localized to caveolae, which are cholesterol-rich microdomains and different transduction of signal located in the plasma membrane. Endothelial cells and HDL promote the activation of eNOS through the binding of apoA-I to the SR-BI receptor *(Yuhanna et al., 2001)*. This link leads to an intricate cascade activation of kinases (Akt/MAPK) *(Mineo et al., 2003)* which increase ceramide levels *(Li et al., 2002)*, leading to phosphorylation and activation of the eNOS enzyme. *In vivo*, HDL infusion synthetic (sHDL) in hypercholesterolemic subjects has led to an increase in bioavailability of NO resulting in the recovery of endothelial functionality *(Spieker et al., 2002)*. Finally, HDL appear to be involved in the reduction of production of reactive oxygen species by eNOS in dysfunctional endothelial cells *(Terasaka et al., 2008)*.

Prostacyclin (PGI_2) is a powerful vasodilator produced by endothelial cells that relax the smooth muscle cells. Arachidonic acid is the substrate that is used by cyclooxygenase (Cox) to produce prostacyclin and derive from phospholipids or cholesterol esters present in circulating lipoproteins. It has been observed that when endothelial cells are treated with HDL you have a dose-dependent increase in prostacyclin release *(Fleisher et al., 1982)*. Cholesterol esters in HDL are an important source of arachidonic acid for the production of PGI_2 *(Pomerantz et al., 1985)*, so it has been hypothesized that esterified cholesterol is internalized after the link between HDL and the SR-BI receptor, thus facilitating the production of NO and PGI_2. It was initially thought that HDL increased the expression of Cox-2 through the activation of the NF-kB transcription factor *(Cockerill et al., 1999)*; on the contrary, it has been shown that the factor that increases the expression Cox-2 is the MAPK, which is triggered by the binding of HDL with the receptor SR-BI *(Mineo et al., 2003; Vinals et al., 1999)*. HDL appear to be able to associate with circulating prostaglandins and stabilize them, thus prolonging their anti-atherogenic effect *(Morishita et al., 1990)*.

Anti-inflammatory properties

Damage to vascular endothelium and pro-inflammatory cytokines act as stimuli for the expression of cell adhesion molecules such as VCAM-1, ICAM-1, E-selectin and P-selectin by the endothelium; these molecules recall monocytes and other circulating leucocytes by inducing their adherence to the vessel wall and promoting their transmigration in the intimae.

In endothelial cell cultures, HDL negatively regulate the expression of cytokine-induced CAMs *(Barter et al., 2002).* The initial hypothesis was the inhibition of the activity of the sphingosine kinase and the nuclear translocation of the nuclear transcription factor kB (NF-kB), an essential prerequisite for the expression of CAMs in response to a pro-inflammatory signal *(Xia et al., 1999).* Recently, however, the re-expression of CAMs has been associated with the activation of an SR-BI dependent pathway and the consequent increase in the level of the enzyme 3-beta-hydroxysteroid-delta24-reductase *(McGrath et al., 2009)*, although the action mechanism of HDL has not yet been fully understood. Synthetic HDL particles (sHDL), containing several apolipoproteins, have proved effective in modulation of CAMs expression, demonstrating that the protein composition of HDL is not detectable for the regulatory effect *(Gomaraschi et al., 2008).* The lipid composition, the length and degree of unsaturated fatty acid residues, on the other hand, significantly influences the ability of HDL to regulate the expression of CAMs *(Baker et al., 2000).* To confirm this, two groups of subjects were exposed to a diet rich in saturated fatty acids or a rich amount of polyunsaturated fatty acids; only the HDL isolated from plasma of subjected exposed to a diet rich in polyunsaturated fatty acids were associated with a reduced expression of CAMs *(NIcholls et al., 2006).* The contribution of HDL form and dimension of HDL to the anti-inflammatory activity has been assessed by several studies: spherical HDL are more effective than discoidal ones (*Baker et al., 1999)* and HDL_3 are more effective than HDL_2 *(Ashby et al., 1998)*.

Antioxidant properties

The onset and progression of atherosclerotic lesions is strongly correlated with the accumulation and subsequent oxidation of LDL in the arterial wall; these processes play a key role in atherosclerosis *(Navab et al., 2004).* Oxidized LDL (oxLDL) have reduced affinity for their liver receptor (LDLR) but are easily picked up by scavenger receptors present on

macrophages. These turn into foam cells and promote the progression of atheromatous plaque *(Navab et al., 2001).* The oxidative changes of lipids, and especially those of LDL, significantly affect the destabilization of the plaque and its eventual break. Molecules and enzymes involved in *in vivo* lipoprotein oxidation include NADPH oxidation, nitric oxide synthase, lipoxygenase and reactive oxygen species such as O_2-superoxide, hydrogen peroxide and radical oxydrile OH• *(Stocker et al., 2004).*

HDL can exert a powerful antioxidant effect through different mechanisms of action (*Tabet et al., 2009).* They accept oxidized fatty acids from cell membranes and LDL according to a process that depends on the lipid stiffness of the surface of the HDL *(Navab et al., 2004).* HDL_3, which are poor of lipid and rich in apoA-I, are more effective in accumulating and inactivating oxidized lipids in relation to HDL_2 *(Zerrad-Saadi et al., 2009).* Oxidized lipids in HDL can be degraded by antioxidant enzymes present within these lipoproteins (PON-1, PAF-AH and LCAT) or reduced by redox reaction with apoA-I and apoA-II, which oxidize the methionine residues *(Tabet et al., 2009).* Once reduced, lipids are transported to the liver where they are then metabolized. HDL carry several antioxidant enzymes in circulation such as PON, a calcium-free that hydrolyzes lipid peroxides by preventing LDL oxidation, and PAF-AH, an A2 calcium-independent that hydrolyze phospholipids to oxidized residues *(Mackness et al., 2000; Marathe et al., 2003)*. The enzyme LCAT also has antioxidant activity and it can both prevent the formation of lipid peroxides in LDL and degrade oxidized phospholipids *(Goyal et al., 1997; Vohl et al., 1999)*. Different studies have shown the importance of HDL and their antioxidant effect in the prevention of atherosclerosis. The antioxidant potential of HDL appears to be positively related to their density, the HDL_3c, smaller and denser, are in fact particularly rich in PON-1 compared to other subclasses, thus showing the maximum antioxidant effect *(Davidson et al., 2009)*.

Antitumoral potential of HDL

Recent studies are highlighted the possible protective role of HDL in prostate cancer development, despite conflicting data. The REDUCE study *(Danilo et al., 2012)* reveals that both total cholesterol and HDL cholesterol are associated with an increased risk of PCa. It is known that cancer cells, especially hormone-dependent ones, express high levels of SR-BI receptor on their plasma membrane and that SR-BI hyperexpression in cancer cells is a cell survival mechanism. since they need cholesterol for proliferation and production of

hormones. However, in the same study it was shown that patients with high plasma levels of HDL-C had reduced prostate inflammation. This observation was further reinforced by a meta-analysis of randomized controlled studies with therapies that alter lipid profiles, suggesting an inverse relationship between plasma levels of HDL-C and the incidence of cancer development. For each increase of 10 mg/dl of HDL-C, the risk of incidence decreased by 36% *(Jafri et al., 2010)*. However, the mechanisms by which HDL carry out their effect are not known. Anti-inflammatory activities, antioxidants activities and the effects of reducing cell cholesterol can be hypotheses.

Some studies in cancer mice report that apoA-I has protective capabilities against tumor development. In these studies, it has been found that in transgenic mice for human apoA-I lung cancer is low, while for heterozygous mice it is intermediate and for knock-out mice it is consistent *(Zamanian-Daryoush et 2013)*. Similarly, in a mouse model of ovarian cancer, the expression of the transgene apoA-I reduced the tumor volume and led to a significant increase in the survival of the animal *(Su et al.,2010)*. In other studies, on knock-out mice for the gene that encodes for apoA-I, the human apoA-I was injected subcutaneously, and later cancer cells was inoculated. It has been observed that apolipoprotein not only prevents the progression of the tumor, but also reduces its size *(Zamanian-Daryoush et al., 2013)*.

Analysis conducted on smoking males who were treated with alpha-tocopherol and beta-carotene showed that there is an inverse correlation between HDL cholesterol (HDL-C) levels and the risk of lung, prostate and liver cancer *(Ahn et al., 2009)*. In accordance with these results, apoA-I levels were also observed to be reduced in patients with early-stage ovarian cancer compared to normal patients, and for this reason apoA-I was identified as a tumor biomarker along with other markers *(Kozak et al., 2005; Zhang et al., 2004; Kim et al.,2012)*.

Mimetic peptides of apoA-I

Based on the role of HDL in reverse cholesterol transport, particularly as cell cholesterol efflux acceptors, several synthetic HDL (sHDL) were developed to stabilize atherosclerotic plaques. These sHDL are cholesterol-free and consist of a double phospholipid layer containing apoA-I, recombinant or purified by plasma, or apoA-I mimetic peptides. Studies conducted with apoA-I or the mimetic peptides L-4F, L-5F and D-4F have shown that treating mice with these proteins can reduce both tumor volume and angiogenesis.

Mimetic peptides of apoA-I were found to have protective activities against both cardiovascular and cancer diseases. These molecules decreased the angiogenic capacity of endothelial cells, as they are able to significantly decrease the production of the vascular endothelial growth factor (VEGF) induced by LPA, through inhibition of the HIF-1 factor, but also interfering in the signal cascade triggered by VEGF in endothelial cells *(Gao et al., 2011; Gao et al., 2012)*. In addition, these peptides are able to inhibit the vitality and proliferation of ovarian cancer cells by reducing oxidative stress in these cells by producing antioxidant enzymes, but also inhibiting growth factors that induce proliferation, migration and cell invasion, and the formation of vessels by endothelial cells *(Gao et al., 2011)*. *In vivo*, the apoA-I mimetic peptide is used in many studies in mice with atherosclerosis and has been shown to have both anti-inflammatory and antioxidant activity, as well as the ability to enhance reverse cholesterol transport *(Navab et al., 2010; Getz et al., 2011; Ditiatkovski et al., 2013)*. ApoA-I has been tested to evaluate the anti-cancer activity. Results showed that it had antitumoral activity in multiple cancers, including ovarian cancers *(Su et al., 2010; Ganapathy et al., 2012)*. In addition, mimetic peptides are used for ovarian cancer by suppressing the activity of LPA and oxidized lipids, which act as tumor growth factors and as promoters of angiogenesis. These peptides are also able to inhibit angiogenesis. Mimetic apoA-I acts with different action and the impact on the tumor environment is profound and multiple. Effects on the tumor environment include both the reduction of angiogenesis resulting in a reduction in tumor volume and metastatic capacity, as well as the increase in plasma levels and intratumor accumulation of tumor-associated macrophages (TAMs) with the M1 anti-tumor phenotype, which are able to eliminate cancer cells *(Zamanian-Daryoush et al., 2013)*.

Aim

HDL are anti-atherogenic lipoproteins, since they prevent the development and progression of atherosclerotic plaques in the arterial wall. The atheroprotective effect of HDL is mainly linked to their central role in the RCT, to the maintenance of endothelial homeostasis and to their antioxidant and anti-inflammatory properties. HDL can act on different cell types involved in the atherosclerotic process such as monocytes, macrophages, endothelial cells and lymphocytes.

In prostate cancer, inflammatory molecules and oxidative stress promote growth, tumorigenicity and neoangiogenesis of epithelial cells, progressively bringing the tumor from a quiescent form to an aggressive growth. In addition, cancer cells need cholesterol to support their high cell growth rate. Moreover, in hormone-dependent tumors, such as PCa, cholesterol can act as a precursor for the local biosynthesis of hormones and favours the survival of cancer cells. Other cholesterol metabolites, such as 27-hydroxycholesterol, also contribute to tumor proliferation by increasing the transcription of androgen receptors. Cancer cells can synthesize the cholesterol needed for proliferation or they can take it from lipoproteins in the tumor microenvironment, such as VLDL and LDL.

HDL could reduce the proliferation and tumorigenicity of these cells through the antioxidant activity, anti-inflammatory activity, and modulation of cholesterol metabolism.

Aim of the project is to study whether plasma-derived HDL could affect PCa cell proliferation through the above-mentioned mechanisms. To this aim, HDL will be tested for their ability to modulate cell cholesterol content, oxidative stress, and the production of inflammatory molecules in normal and tumor prostatic epithelial cells. Then, the impact of these effects on PCa cell proliferation will be tested. Since HDL are not suitable for drug development due to safety concerns, aim of the study will be to verify whether synthetic HDL, made of apoA-I and phosphatidylcholine, retain the effects of plasma-derived HDL on PCa cells.

Materials and methods

Cell lines

Different cell lines were used: LNCaP and PC-3 that are two different human epithelial cell models of prostatic tumor and the human normal prostate epithelial cell line PNT2 (Figure 12). LNCaP are AR positive and sensitive and were shown to be tumorigenic in nude mice with a low metastatic potential *(Sobel et al., 2005)*. On the contrary, PC-3 are AR-null and display an androgen-independent growth; they were shown to be tumorigenic with a high angiogenic and metastatic potential in nude mice *(Sobel et al., 2005)*. SV40-immortalized PNT2 are non-tumorigenic human prostatic epithelial cells *(Berthon et al., 1995)*. Tumor cell lines were purchased from ATCC (VA, USA) and PNT2 were purchased from Sigma-Aldrich (Germany). Cell lines were maintained in RPMI 1640 supplemented with 5% (PC-3) or 10% (LNCaP and PNT2) of fetal bovine serum, 1% of penicillin/streptomycin and 2 mM of L-glutamine and they were preserved at 37 °C in a humidified atmosphere with 5% CO_2. Confluent cells were harvested with 0.05% trypsin/0.02% EDTA and seeded in petri dishes or multiwell plates, as requested for each experiment. Cell media, supplements and sterile disposables were purchased from EuroClone (Italy).

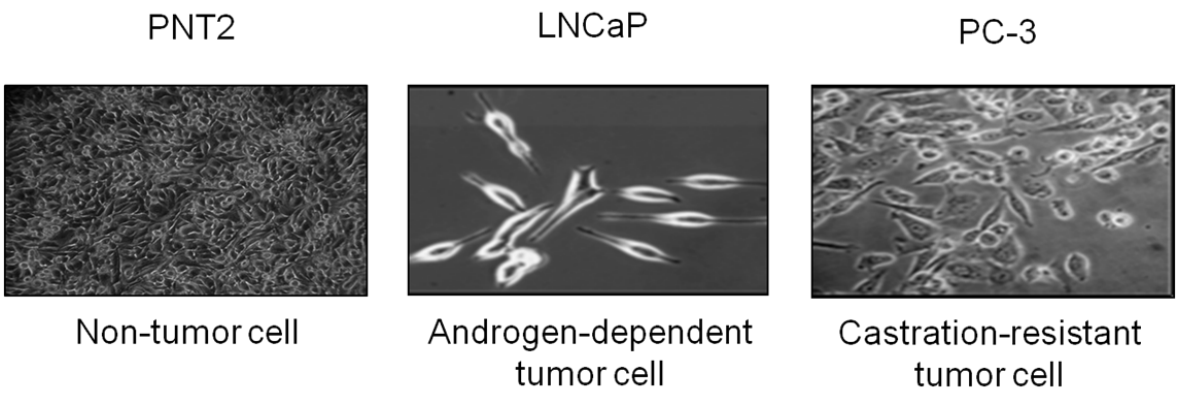


Figure 12. Prostate non-tumor cells PNT2, prostate cancer cells androgen-dependent phenotype LNCaP and castration-resistant phenotype PC-3.

Lipoproteins

Lipoproteins HDL (d 1.063-1.21 g/ml) and LDL (d 1.020-1,063 g/ml) were isolated by control subject serum (d 1,006 g/ml). Lipoproteins were separated by sequential

ultracentrifugation. Initially, an amount of KBr was added to the serum to reach the density of 1,020 g/ml. The solution was stratified with a KBr solution that has the same density in special tubes. As a result of the ultracentrifuge process, lipoproteins with density < 1,020 g/ml were floated, while LDL and HDL remain in the lower portion of the tube. The lower fraction was collected and KBr was added to reach a density of 1,063 g/ml. The solution was stratified with a KBr solution that has the same density. After the ultracentrifuge process, lipoproteins with density < 1,063 g/ml were floated, such as LDL, in the upper portion of the tube, while HDL remain in the lower portion. Both parts were collected, and for HDL the density was corrected to 1.21 g/ml. The solution was stratified with a KBr solution that had the same density and as a result of the ultracentrifuge process, HDL were floated in the upper portion. Finally, HDL and LDL were stratified with a KBr solution with density 1,25 g/ml and 1,080 g/ml respectively to eliminate impurities, ultracentrifuge causes the lipoprotein floating in the upper area of the tube and they were collected. Then, the lipoproteins were dialyzed in physiological buffer to eliminate the KBr solution in the samples. HDL and LDL lipoproteins were dialyzed against sterilized saline immediately before use and dosed by Lowry's method (Lowry et al, 1951) and filtered.

Purification of apoA-I from serum

Apolipoprotein A-I was prepared by splitting the protein portion of HDL by chromatography (Brewer, et al., 1986). The HDL were dialyzed with bicarbonate buffer (NH_4HCO_3 5mM at pH-7.9) containing EDTA at 0.01%, lyophilized and delipidated with a diethyl-ether/methanol mixture 5/1 in volume. Subsequently, the apolipoproteins obtained were solubilised in tampon Tris-HCl 0.2 M to pH 7.4 containing 6 M guanidium chloride, then split on a chromatic column packed with Sephacryl S-300 HR (2.6 x 300 cm), balanced with Tris-HCl buffer at pH 7.4 containing 4 M guanidium chloride. The same buffer was used to elute the apolipoprotein fraction to a flow of 0.8 ml/min. Fractions containing apoA-I were collected, concentrated, and purified by chromatography under the same conditions. The purified apoA-I was dialyzed to the EDTA-containing bicarbonate buffer at 0.01% at pH 7.9, aliquoted and stored at -80°C. Finally, the purity of the protein was verified by electrophoresis on 16% polyacrylamide gel (SDS-PAGE), conducted according to the technique described by Schagger & Von Jagow (1987). Prior to use, the protein was

dissolved in 4 M guanidium chloride and dialyzed to Tris 10 mM buffer at pH 8.0, containing NaCl 150 mM, EDTA 1 mM and NaN_3 1 mM.

ROS assay

PNT2, LNCaP and PC-3 cells seeded in 24-well black plates were incubated with HDL, sHDL or apoA-I at 0.5 mg/ml up to 16 hours in RPMI 1640 (supplemented with 1% bovine serum albumin for LNCaP). Cells were then washed with PBS and loaded with 3 µM 2',7'-dichlorofluorescein (Life Technologies, USA) for 30 minutes. After RPMI 1640 replenishment, cells were stimulated or not with 0.5 mM H_2O_2 for 1 hour and ROS production was measured by fluorescence on a Synergy H1 multi-mode reader equipped with the Gen5 software (BioTek, USA). To evaluate the role of AR signalling in ROS generation and HDL antioxidant effect, in separate experiments LNCaP were maintained in RPMI 1640 without phenol red and incubated overnight with the AR antagonist bicalutamide (100 nM, Sigma-Aldrich). To evaluate the role of cell cholesterol content on HDL antioxidant effect, cells were pre-treated overnight with LDL (50 µg/ml) or for 1 hour with 2.5 mM beta-methylcyclodextrin (βMCD, Sigma Aldrich, Germany).

For each sample, fluorescence was normalized by the protein concentration of the total cell lysate, measured by the micro-bicinchoninic acid assay (Thermo Scientific, USA).

A standard HDL preparation from pooled plasma was tested in each experiment for inter-assay data correction.

Cell cholesterol content

To evaluate cell cholesterol content, LNCaP and PC-3 cells were seeded in 12-wells plate at a density of 0.1 million cells/well in growth medium. After three days, the growth medium was replaced with stimuli medium RPMI 1640 supplemented with 1% Penicillin/Streptomycin, 1% L-glutamine and incubated for 1 hour with HDL (0.5 mg protein/ml) or LDL at the same cholesterol content (50 µg/ml protein). Then cells were washed with PBS and lysed with 1% sodium cholate in water and 10 U/ml of DNase (Sigma Aldrich, Germany) overnight at room temperature. Cholesterol was measured by fluorescence using the Amplex Red Cholesterol Assay Kit (Sigma Aldrich, Germany),

according to the manufacturer instructions *(Calabresi et al., 2009)*. For each sample, cholesterol concentration was normalized by the protein concentration of the total cell lysate, measured with the micro-bicinchoninic acid assay (Thermo Scientific, USA).

To evaluate the effect of HDL on cell cholesterol content, LNCaP and PC-3 were loaded of cholesterol with LDL 50 µg of protein/ml and then cells were treated for 1 hour with 0.5 mg/ml of HDL or 0.5 mg/ml of apoA-I. Then cell cholesterol content was measured as before.

To evaluate the presence of free-cholesterol in cell membrane, cells were treated with 2.5 mM of beta-methyl-cyclodextrin for 1 hour (Sigma Aldrich, Germany), then cell cholesterol content was evaluated in the same way.

Cell cholesterol content was also evaluated in PC-3 cells, after treatment with proteasome inhibitors, such as MG132 (Sigma Aldrich, USA) used at concentration of 50 µM for 4 hours or bortezomib (Cell Signaling) used at concentration of 5 nM for 8 hours.

Finally, to evaluate the possible role of SR-BI in the lack of cholesterol efflux to HDL, cell cholesterol content was measured in PCa cells after silencing of SR-BI.

Gene expression

PNT, LNCaP and PC-3 cells were seeded in 12-wells plate to a density of 0.4 million cells/well in growth medium. The cells were then collected with TRIzol Plus RNA purification (Life Technologies) for the extraction of messenger RNA from cellular lysates. The cellular lysates were left for 10 minutes at room temperature and then 50 µl of chloroform was added and samples were centrifugated at 8000 rpm for 10 minutes (Beckman Optima TL-100). Centrifuge allows to the RNA to be separated into a higher transparent phase that was later saved and measured in order to add equal amounts of ethanol (70%). After adding ethanol, the solution was transferred to the cartridge tube, centrifuged for 15 seconds at 12000 rpm and washed with 700 µl of Wash Buffer 1. Then an additional 15 second centrifuge was executed, and the solution was washed twice with 500 µl of Wash Buffer 2, centrifuged for 15 seconds and re-centrifuged for 1 minute dry. The cartridge has been moved to a sterile tube and 30 µl of H_2O was added. After 1 minute, the solution was centrifuged for 2 minutes. RNA was eluted in a sterile tube and then dosed by measuring the absorbance to

260 nm (NanoDrop 2000). To check for no contamination from proteins or solvents, the absorption was measured at 280 and 230 nM respectively.

The synthesis of cDNA was performed via the iScript™ (BIO-RAD Laboratories) kit. Subsequently, 0.8 µg of RNA was retrotranscribed by additional 4 µl of Mix and 1 µl of enzyme. The mixture was inserted into the MJMini (BIO-RAD) thermocycler. Finally, the solution containing cDNA was diluted with H_2O of the kit.

Real Time PCR experiments were performed using the iTaq Universal SYBR Green kit in the MJMini thermocycler and the OPTICON MONITOR 3 (BIO-RAD Laboratories) software. The reaction mixture used for each sample was prepared with 10 µl of Mix, 0.6 µl of primer forward, 0.6 µl of primer reverse, 7.3 µl of H_2O and 1.5 µl of cDNA. The expression of ABCA1, LDLR, LRP1 and HMGCoAR were evaluated. The GAPDH method was used as a reference gene of constitutive expression.

Protein expression

PNT2, LNCaP and PC-3 cells were seeded in 24-wells plate to a density of 50,000 cells/well in growth medium. Cells were collected when confluent and lysed in a Tris Buffer 20 mmol/L with SDS at 4%, glycerol at 20%, 1 mmol/L of EDTA, 1 mmol/L of orthovanadate sodium, 1 mmol/L of Sodium Fluoride, 1 g/L of leupeptin, 1 mmol/L of benzamidine, 10 g/L of trypsin inhibitor, 1 mmol/L of PMSF and 0.5 mmol/L of DDT. Cell debris was removed by centrifugation and protein concentration was determined by the micro-bicinchoninic acid assay (Pierce). Samples were loaded, proteins were separated by 10% SDS-PAGE and transferred on a nitrocellulose membrane. Membranes were incubated either with ABCA1, ABCG1, SR-BI (Novus Biologicals, USA), LDLR (Invitrogen), LRP1, HMGCoAR (abcam) and alpha-tubulin (Sigma Aldrich, Germany). Bands were visualized by ECL (GE Healthcare Biosciences, Sweden) and band densities were evaluated with a GS-690 Imaging Densitometer equipped with the Multi-Analyst software (Bio-Rad Laboratories).

Protein expression of ABCA1 in cell membrane

To assess the membrane expression of ABCA1, LNCaP and PC-3 cells were seeded in 10 cm^2 petri dishes in growth medium and cells were collected when reached the confluence. Subsequently, cells were lysated in RIPA Buffer with 150 nM NaCl, 1% Igepal CA-630, 0.5%

Na Desoxyn, 0.1% SDS in 50 mM TRIS at pH 8.0 and increased to 2 ml final volume with RIPA Buffer. The solution thus obtained was centrifuged for 30 minutes at 4 °C at 20000 rpm. Subsequently, the supernatant was removed, and pellet was resuspended in 800 µl of RIPA Buffer + 4 mg/ml of dodecyl-beta-maltoside, which was incubated for 1 hour at 4 °C and again centrifuged for 1 hour at 4 °C at 20000 rpm. Finally, the supernatant containing fraction of membrane was collected. The micro-bicinchoninic acid assay (Pierce) was performed to determine the protein concentration, while to assess the protein expression of ABCA1 in membrane, the sample was loaded onto the gel and the Western Blot analysis was performed.

Immunofluorescence

PC-3 cells were seeded at a density of 80,000 cells/well in growth medium in 6-wells plate, each containing a sterilized slide. When cells reached a confluence of 20%-50%, cells were treated. To test the possible involvement of protein degradation pathway, cells were treated with: MG132 (Sigma-Aldrich, USA) 50 µM for 4 hours or bortezomib (Cell Signaling) 5 nM for 8 hours that are proteasome inhibitors; Chloroquine diphosphate that is an inhibitor of lysosomal proteases (Sigma-Aldrich, USA) 100 µM for 8 hours; Calpeptin that is a calpain inhibitor (Sigma-Aldrich, USA) 30 µg/ml After treatments medium was removed, and cells were fixed and permeabilized in 100% cold methanol for 10 minutes at room temperature. In other experiments cells were fixed and unpermeabilized with 4% of paraformaldehyde for 5 minutes at room temperature. Then slides were washed with PBS quickly and incubated with PBS at 10% FBS for 20 minutes to block non-specific antibody sites. At the end of the time, cells were co-incubated with primary antibodies diluted 1:100 in PBS 0,1% Tween20 at 1% BSA and incubated o/n at 4° C. Cells were stained with ABCA1 rabbit Polyclonal Primary Antibody (ThermoFisher, USA) and with alpha-tubulin mouse Polyclonal Primary Antibody (Sigma-Aldrich, USA). The day after, slides were washed three times, to remove the unbound antibody, with PBS for 5 minutes each. Then cells were co-incubated with secondary antibodies: anti-mouse AlexaFluor-488 (Invitrogen) and anti-rabbit Rhodamine conjugated (Invitrogen) both diluted 1:500 in PBS with 0,1% Tween20 at 1% BSA for 1 hour at room temperature. Then, slides were washed to remove the unbound antibody for five times with PBS and finally slides were washed in water. At the end 1 drop

of mounting medium containing DAPI (ThermoFisher, USA) was added on each slide and they were mounted on a slide. Then they were preserved at -20° C until the observation. The fluorescence images were obtained with 60× objective using a confocal microscope Nikon A1 (Zeiss). Intensity of fluorescence was measured with ImageJ software and each measurement was corrected to area and density.

Silencing of genes

PC-3 cells were seeded in 12-well plates and transfected with 100 pmol of siRNA against SR-BI, ABCG1 or noncoding (scrambled) siRNA for 48 hours using the OptiMEM/Lipofectamine 2000 system (Life Technologies, UK), according to the manufacturer's protocol. siGenome ON-TARGETplus SMARTpool were used (Dharmacon, ThermoScientific, USA). Cells were then washed with PBS and lysed with 1% sodium cholate in water and 10 U/ml of DNase (Sigma Aldrich, Germany) overnight at room temperature. Finally, cell cholesterol content was measured as described above. Silencing efficiency was evaluated by western blotting for SR-BI and alpha-tubulin, as described above.

Cell proliferation

LNCaP and PC-3 cells were seeded at 150,000 and 100,000 cells/well respectively in 6-wells plate in growth medium. After 48 hours of incubation, a change medium was made with RPMI 1640 without phenol red, to avoid estrogens activity, containing 10% or 5% respectively of charcoal-stripped FBS, to avoid presence of hormones, and cells were incubated for 48 hours. Then another change medium was made with RPMI 1640 without phenol red supplemented with 10% or 5% with charcoal-stripped FBS respectively, and cells were treated with LDL (20 µg protein/mL) for 24 hours and then with HDL for other 48 hours (0.5 mg protein/mL).

At the end of the experiment, cells were collected and counted using the Trypan Blue dye in Burker's chamber. Cell growth was further analyzed by the MTT assay (Promega, USA), according to manufacturer instructions. After 4 hours at 37 °C, absorbance at 570 nm was measured with the Synergy H1 multi-mode reader equipped with the Gen5 software (BioTek, USA).

Cell proliferation was also measured in PC-3 cells, after co-treatment with LDL and proteasome inhibitor bortezomib (Cell Signaling Technology), used at concentration of 5 nM for 8 hours, followed by HDL treatment for 48 hours as before.

Cell cycle analysis

Flow cytometry was used to analyse cell cycle distribution. After trypsinization, LNCaP and PC-3 were re-suspended in 70% cold EtOH (700 µL) and fixed on ice for 15 minutes. Cells were then centrifuged, the pellet was washed with cold PBS Ca^{2+}/Mg^{2+} and re-suspended in a solution containing 40 µg/mL of RNAse and propidium iodide (5 µM). Samples were kept in the dark for 15 minutes, and the fluorescence of individual nuclei was measured. Data acquisition was performed in a NovoCyte flow cytometer (ACEA Biosciences, USA) using NovoExpress 1.0.2 software. A minimum of 10,000 events/sample were counted. The number of cells in G0/G1, S, and G2/M phases was expressed as percentage of total events.

Colony formation assay

PC-3 and LNCaP cells were seeded in 6-well plates at a density of 200 cells/well in growth medium. The day after medium was changed and some wells were treated with 0.5 mg/ml of protein of HDL for 48 hours, after the first 24 hours some wells of PC-3 cells were also treated with 50 pM of bortezomib (Cell Signaling Technology). At the end of the 48 hours medium was changed. In the following two weeks only medium was changed until the end of two weeks. After 14 days, colonies were counted. First, the cells are fixed in 100% cold methanol for 7 minutes. Next, methanol was removed until the evaporation. At this point, Giemsa dye solution (Sigma-Aldrich, Switzerland) was added to each well, previously diluted 1:20 in water, for 45 minutes. Finally, the staining was removed, and each well was washed with deionised water. Then, colonies formed by 50 or more cells were counted with optical microscopy. The efficiency of colonies formation was calculated as a parameter to assess the proliferation.

Statistical analysis

The results are expressed as mean ± standard deviation, except where specified. The statistical analysis was performed with the Sigmaplot 12.0 program. Differences between

treatment groups were assessed using *t*-test or variance analysis (ANOVA). *P* values < 0.05 were considered statistically significant.

Results

Modulation of oxidative stress and impact on the proliferation of PCa cells

Oxidative stress in PCa cells

Basal oxidative stress was evaluated in PCa cells, LNCaP and PC-3, compared to non tumor cells, PNT2, using fluorescent probe. Reactive oxygen species (ROS) were increased in PCa cells and the observed increase was proportional to the aggressiveness of the phenotype. In particular level was 1.4 fold higher in LNCaP and 2.6 fold higher in PC-3 compared to that observed in PNT2 cells (Figure 13).

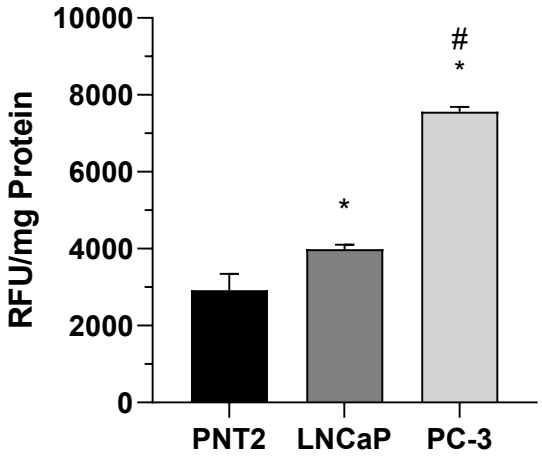


*Figure 13. Basal oxidative stress in PNT2, LNCaP and PC-3 cells. Oxidative stress evaluation is expressed as relative fluorescent units (RFU) normalized by the protein concentration of total cell lysate, mean±SD. *P<0.05 vs PNT2, #P<0.001 vs LNCaP.*

Then, the antioxidant effect of HDL in LNCaP and PC3 cells was evaluated measuring the ROS production induced by a pro-oxidant stimulus, such as H_2O_2.

Basal ROS production, indicated by black bars (Figure 14), in LNCaP was significantly reduced when incubated with HDL overnight (-28%, p=0.007) or only for 1 hour before ROS detection (27%, p=0.006). Basal oxidative stress in PC-3 cells was also reduced by HDL given 1 hour before ROS detection (-31%, p<0.001), but not for a longer incubation, probably due to the higher ROS production in these cells.

In order to mimic a pro-oxidant environment, cells were also stimulated with H_2O_2 for 1 hour to evaluate the ability of HDL to inhibit ROS production, indicated by red bars (Figure 14).

The 1.6-fold increment in basal ROS production was induced in LNCaP cells by H_2O_2, but this increase was completely blunted when cells were co-incubated with HDL. Interestingly, ROS increase was again completely blunted when HDL were added overnight or only for 1 hour and then removed before H_2O_2 stimulation. The same experiments were performed in PC-3 cells, the treatment with H_2O_2 induced a 1.3-fold increment in ROS production that again was prevented by all treatments with HDL.

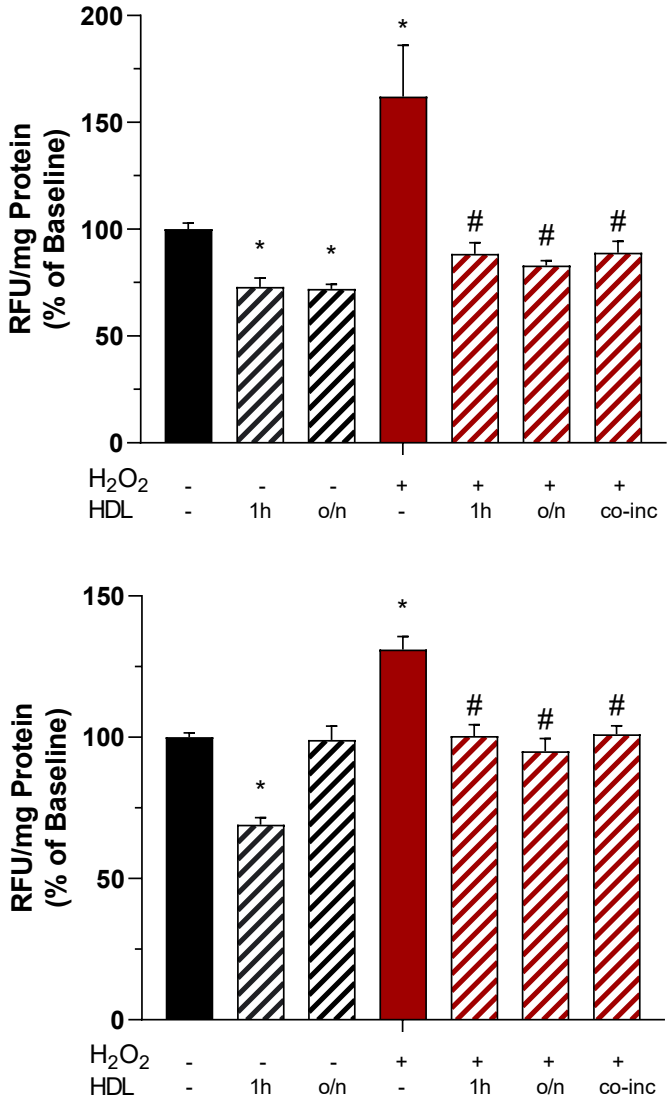


Figure 14. ROS production in LNCaP and PC-3 cell lines was reduced by HDL. LNCaP (Panel A) and PC-3 (Panel B) cells were pre-treated overnight (o/n) or for 1h with HDL 0.5 mg/ml and then stimulated or not with 0.5 mM H_2O_2 for 1h. It was also tested the effect of the co-incubation of HDL with H_2O_2 (co-inc). Fluorescence was used to evaluate ROS

*production. Data are expressed as relative fluorescent units (RFU) normalized by the protein concentration of total cell lysate, mean±SD. *P<0.05 vs control, #P<0.05 vs H_2O_2-stimulated cells.*

Cell proliferation induced by ROS production in PCa cells

PCa cells were exposed to 5 µM H_2O_2 for 72 hours to reproduce a chronic low-grade pro-oxidant environment and the influence on cell proliferation was evaluated (Figure 15). The treatment on LNCaP cells caused a significant increase of cell proliferation rate, with an increase in cell number of 25.3% ($p<0.001$). Cell proliferation rate was completely blunted and comparable to untreated cells through the co-incubation with HDL that significantly reduced H_2O_2-mediated increase of cell count of 23.1% ($p=0.003$). Similarly, cell growth stimulation induced by H_2O_2 on PC-3 caused a significant increase of cell number of 23.3% ($p=0.024$), and this effect was completely blunted by the co-incubation with HDL (-33.6% vs H_2O_2, $p<0.001$). Interestingly, basal cell proliferation showed a decrease when both PCa lines were treated with HDL alone, although the effect was not statistically significant (LNCaP -14.4% vs untreated cells, $p=0.190$; PC-3 -14.7% vs untreated cells, $p=0.131$). MTS assay was used to confirm the data in both cell lines.

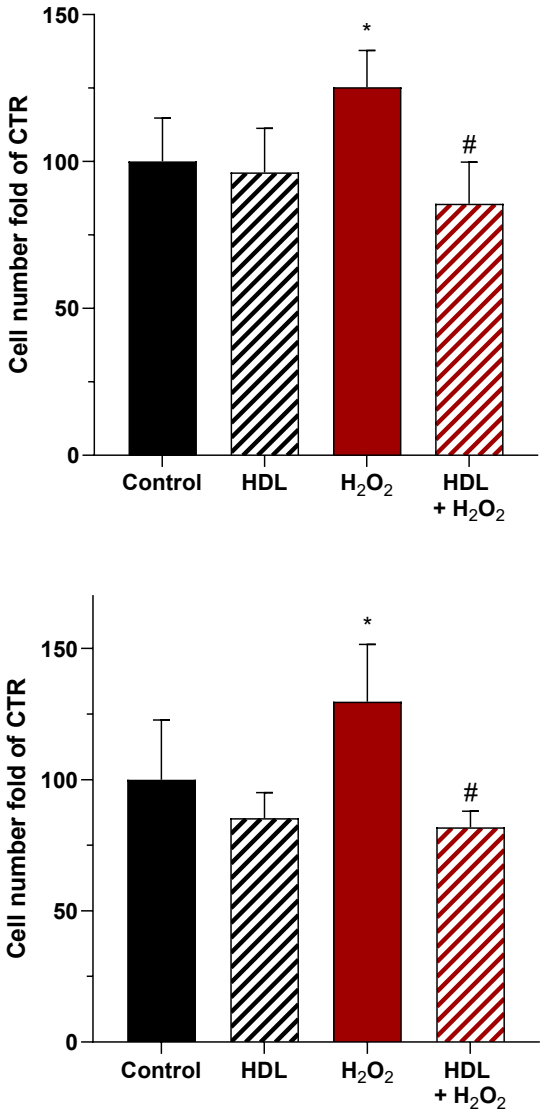


*Figure 15. Cell proliferation induced by H_2O_2 in LNCaP and PC-3 cell lines was inhibited by HDL. LNCaP (Panel A) and PC-3 (Panel B) cells were incubated with H_2O_2 5 µM, HDL 0.5 mg/ml or with a combination of both for 72h. Cells were then harvested and counted using a Burker chamber. Data are expressed as mean±SD. *P<0.05 vs control, #P<0.05 vs H_2O_2-stimulated cells.*

The proliferative effect of H_2O_2 was confirmed by the shift of cell cycle distribution. The incubation of LNCaP with H_2O_2 for 72 hours, in comparison to untreated cells, resulted in reduction of cell percentage in the G0/G1 phase from 60±6% to 51±6%, with a concomitant increase of cells in the G2/M phase (from 9±1% to 19±1%, p<0.001, Figure 16, Panel A). The shift from the G0/G1 towards the G2/M phase was completely blunted using the concomitant incubation with HDL, obtaining indeed similar values of untreated cells (9±1%).

The exposure of PC-3 cells to H_2O_2 for 72 hours also caused an increase of cell percentage in the G2/M phase from 14±4% to 18±1%, and again this rise was completely blunted in the presence of HDL; indeed the co-incubation restored the basal cell cycle profile, with a significant reduction of cells in the G2/M phase (6±1%, p=0.003 vs H_2O_2) and increase of cells in the G0/G1 phase compared to H_2O_2-treated cells (from 52±5% to 67±7%, p=0.035 vs H_2O_2, Figure 16, Panel B).

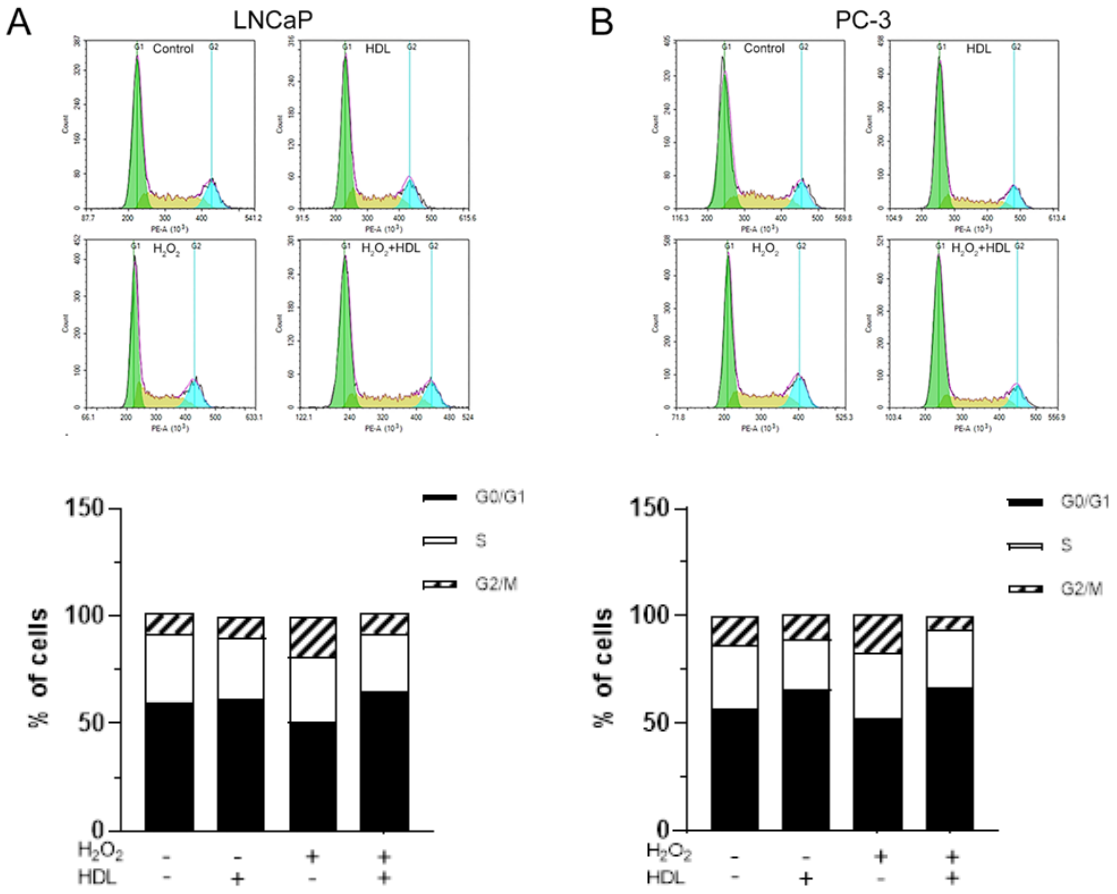


Figure 16. Cell cycle distribution was modulated by HDL in H_2O_2-treated cells. LNCaP (Panel A) and PC-3 cells (Panel B) were treated for 72h with H_2O_2 5 µM, HDL 0.5 mg/ml or with a combination of both. Cells were then subjected to FACS analysis after harvesting and staining with propidium iodide. Each experimental group and cumulative results of the percentage distribution along cell cycle (n=3) are shown in representative histograms.

Synthetic HDL exert antioxidant activity on LNCaP and PC-3 cells

Plasma derived HDL are unsuitable for drug development since they are a heterogeneous family of lipoproteins for safety concern. This problem can be overcome by preparing synthetic HDL (sHDL), composed by the two main component of plasma derived HDL mainly apoA-I and phosphatidylcholine. These particles differ from the majority of plasma derived

HDL since they are discoidal and not spherical particles. sHDL were prepared and tested for their antioxidant effects in two cell models of PCa. sHDL consisted of a single population of particles defined by a diameter of 9.7 nm, with unlipidated apoA-I being undetectable. According to final weight ratio, each sHDL contained 2 apoA-I and 148 POPC molecules per particle (Figure 17).

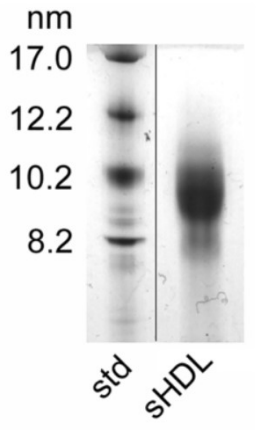


Figure 17. Synthetic HDL. GGE was used to verify the sHDL particle formation and size.

Synthetic HDL made of apoA-I and POPC were used to treat PCa cell lines showing a decrease of ROS production when given in combination with H_2O_2 in both LNCaP (-42%, $p<0.001$) and PC-3 cells (-35%, $p=0.003$) (Figure 18). sHDL were also able to reduce reactive oxygen species production when given for 1h and then removed before to H_2O_2 stimulation; ROS production was indeed decreased by 35% in LNCaP ($p<0.001$) and by 33% in PC-3 cells ($p=0.002$).

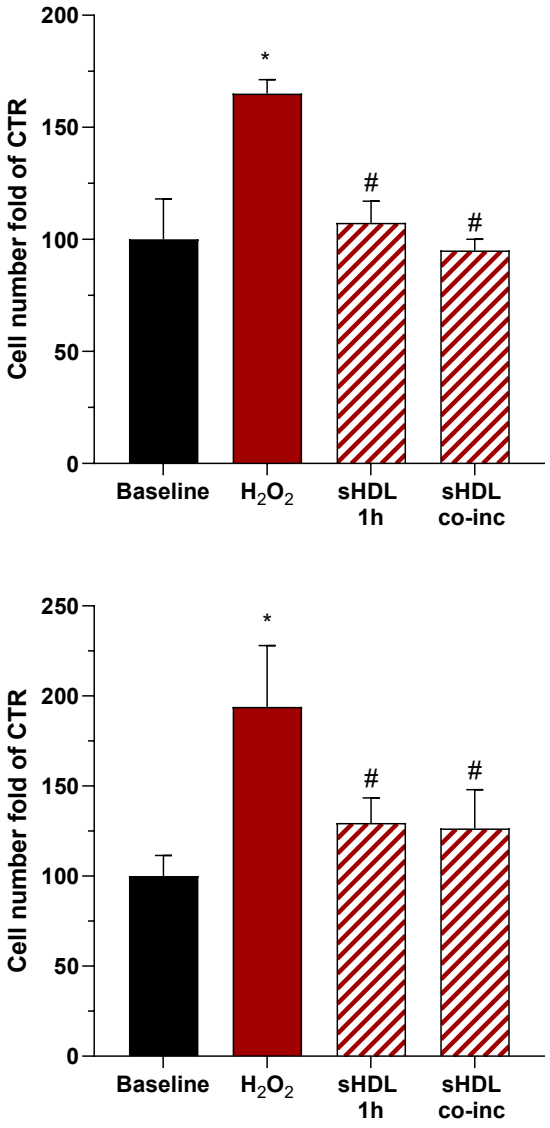


*Figure 18. Synthetic HDL and plasma-derived HDL retain the same antioxidant effect. LNCaP (Panel A) and PC-3 (Panel B) cells were treated with 0.5 mM H_2O_2 for 1h. sHDL were tested at 0.5 mg/ml as a pre-treatment of 1h or as concomitant incubation with H_2O_2 (co-inc). Fluorescence was used to evaluate ROS production. Data are expressed as relative fluorescent units (RFU) normalized by the protein concentration of total cell lysate, mean±SD. *P<0.05 vs control, #P<0.05 vs H_2O_2-stimulated cells.*

Expression of transporters and receptor in prostate cell lines

To address whether transporters and receptors, like ABCG1, ABCA1 and SR-BI, which are well known as mediators of many activities of HDL, were also involved in the antioxidant effect of HDL observed in PCa cells, the expression of these was evaluated (Figure 19).

The data obtained show that the expression of the ABCA1 transporter was significantly decreased in cancer cells compared to non-tumor cells. In fact, ABCA1 was poorly expressed in PNT2 and there was a significant decrease in this expression in both tumor cell lines (-69% in LNCaP and -81% in PC-3). On the other hand, the ABCG1 transporter was

poorly expressed in PNT2, while in the other lines the expression was increased significantly; in particular, a 2.8-fold increase in LNCaP and a 5.8 fold increase in PC-3 were detected. The SR-BI receptor was equally expressed in PNT2 cells without significant changes in the PCa cell lines.

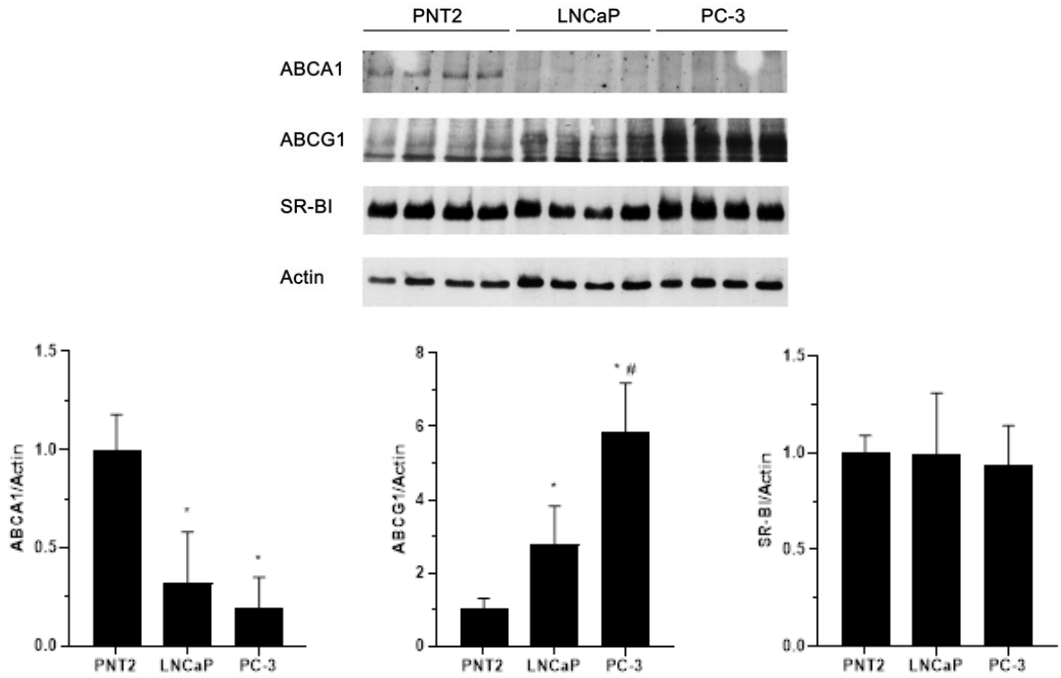


*Figure 19. Protein expression of ABCA1, ABCG1 and SR-BI in tumor and non-tumor prostate cells. Protein expression was analysed via western blot in PNT2, LNCaP and PC-3. For western blot analysis, 40 µg of protein was loaded for each sample. The data are expressed as the ratio between the target signal (ABCA1, ABCG1, SR-BI) and the signal of constitutive expressed actin protein, and were normalized based on the expression in the non-tumor cell PTN2, mean±SD, n=7 * p <0.05 vs PNT2, #p <0.05 vs LNCaP.*

Involvement of transporters and receptor in antioxidant effect of HDL

The possible role of SR-BI and ABCG1 in mediating HDL antioxidant effect was tested by RNA-interference (Figure 20).

The treatment with specific siRNA caused a marked inhibition of SR-BI and ABCG1 protein expression; indeed, SR-BI protein expression was reduced by 77.0 ± 8.9% and ABCG1 by 76.0 ± 8.3%. The severe reduction of SR-BI expression affected neither the ROS levels after H_2O_2 treatment, nor the antioxidant effect of HDL. Similar results were obtained when ABCG1 was silenced. Being ABCA1 almost undetectable in tumour cells, silencing experiments targeting this transporter were not performed.

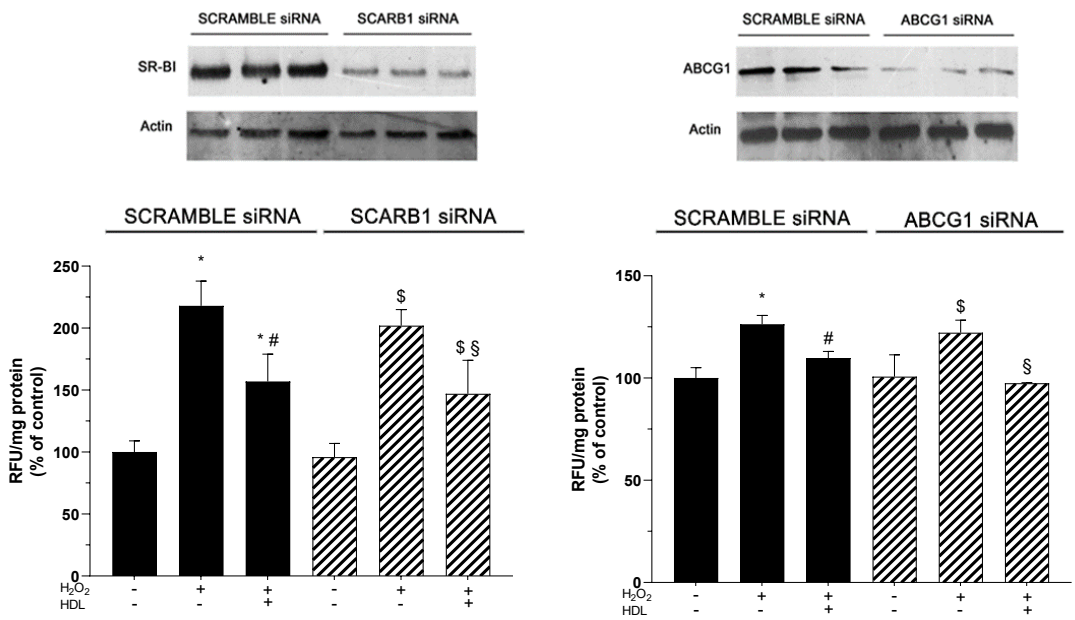


*Figure 20. SR-BI and ABCG1 are not involved in HDL antioxidant effect. Protein expression of SR-BI and ABCG1 was analysed via western blot in PC-3 cells treated with specific or control (scrambled) siRNA. ROS levels in H_2O_2-stimulated PC-3 cells pre-treated with specific or control (scrambled) siRNA. The effect of the co-incubation of 0.5 mM H_2O_2 with 0.5 mg/ml HDL for 1h was tested. ROS production was evaluated by fluorescence. Data are expressed as relative fluorescent units (RFU) normalized by the protein concentration of total cell lysate, mean ± SD, n = 3. *P < 0.05 vs control cells incubated with scrambled siRNA, #P < 0.05 vs H_2O_2-stimulated cells incubated with scrambled siRNA, $P < 0.05 vs control cells incubated with ABCG1 or SCARB1 siRNA, §P < 0.05 vs H_2O_2-stimulated cells incubated with ABCG1 or SCARB1 siRNA.*

Involvement of androgen receptor in oxidative stress production in PCa cells

Finally, since some evidence suggest that androgen receptor activation could be involved in the production of the oxidative stress on these cells, the relevance of the androgen receptor activation in our experimental setting using the antagonist Bicalutamide was tested. The AR antagonist Bicalutamide was used to disclose the role of AR signalling in mediating ROS production and HDL antioxidant effect in LNCaP (Figure 21). Overnight incubation with Bicalutamide did not modify ROS increase after H_2O_2 treatment; in fact, ROS levels in cells pre-treated with Bicalutamide and then stimulated with H_2O_2 for 1 hour were comparable to those of untreated H_2O_2-stimulated cells. HDL retained their antioxidant effect on H_2O_2-stimulated cells even in the presence of Bicalutamide. In fact, when LNCaP were pre-treated with Bicalutamide and then stimulated with H_2O_2, HDL were able to significantly reduce ROS levels as an o/n pre-treatment or as a co-incubation with H_2O_2.

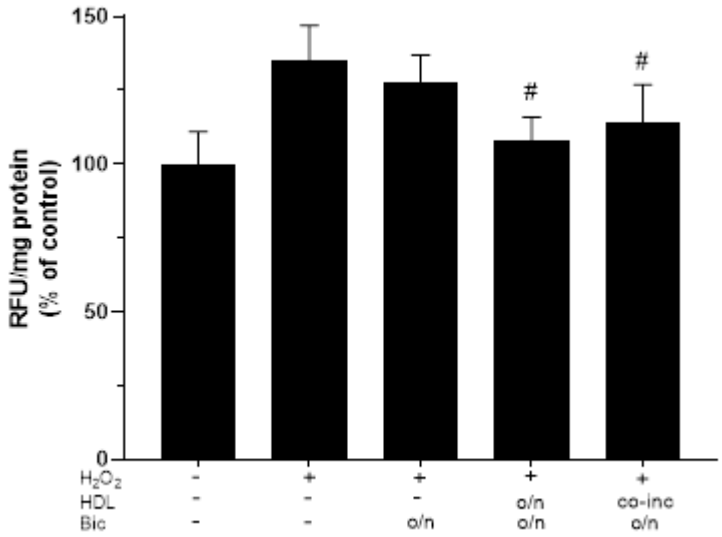


Figure 21. AR activation is not involved in HDL antioxidant effect. LNCaP were treated overnight (o/n) with the androgen receptor antagonist Bicalutamide (100 nM) before stimulation with 0.5 mM H_2O_2 for 1h. HDL at 0.5 mg/ml were given overnight or as co-incubation with H_2O_2 (co-inc) for 1h. ROS production was evaluated by fluorescence. Data are expressed as relative fluorescent units (RFU) normalized by the protein concentration of total cell lysate, mean ± SD, n = 6. #P < 0.05 vs H_2O_2-stimulated cells.

Modulation of cholesterol homeostasis and impact on the proliferation of PCa cells

Expression of genes and proteins involved in cholesterol homeostasis in PCa cells

To evaluate whether in PCa cell lines the receptors, transcription factors and enzymes involved in the synthesis of cholesterol, in the endocytosis of lipoproteins and in the cellular cholesterol efflux process are altered, their gene and protein expressions were evaluated.

LDLR and LRP1

LDLR and LRP1 (LDL receptor-like protein) recognize apoB-100 and apoE, promote the uptake by endocytosis of lipoproteins and consequent supply of cholesterol to cells. Therefore, gene and protein expression of LDLR and LRP1 in the three cell lines was assessed (Figure 22, 23).

Gene expression of LDLR in tumor cell lines was significantly increase. In particular, it was about 3 times higher for LNCaP and about 5 times higher for PC-3 compared to PNT2 cells ($p<0.05$).

The increase in receptor expression has also been confirmed in terms of protein expression. In fact, the LDLR protein levels in PCa cells were significantly increased compared to non tumor cells PNT2.

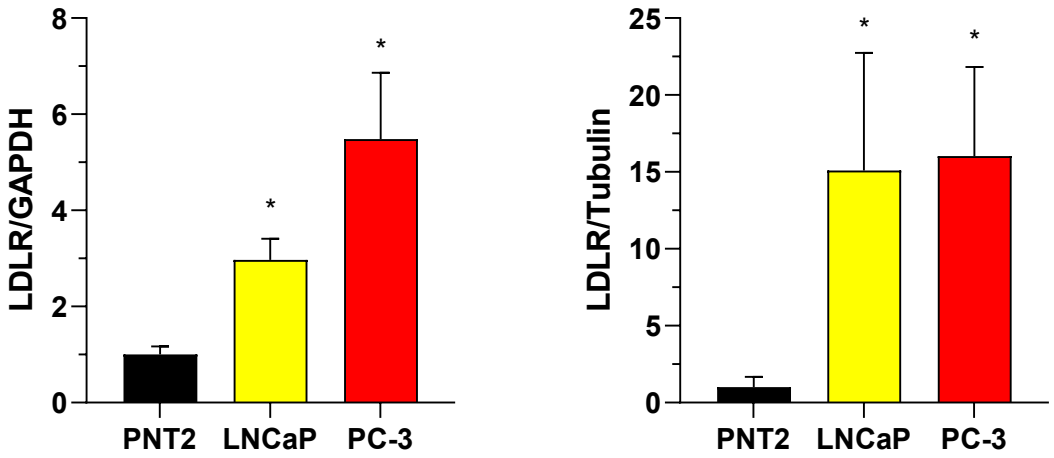


*Figure 22. Gene expression of LDLR in prostate cancer cells compared to PNT2 cells. Gene expression was analysed by Real-Time PCR in PNT2, LNCaP and PC-3. Protein expression of LDLR in PNT2, LNCaP and PC-3 cells. Data are expressed as mean±SD, *p<0.05 vs PNT2. Protein expression was analysed via western blot in PNT2, LNCaP and PC-3.*

Gene expression of LRP1 in tumor cell lines was significantly increase. In particular, it was about 4 times higher for LNCaP and about 7 times higher for PC-3 compared to PNT2 cells ($p<0.05$).

Also, the LRP1 protein levels were significantly higher in both cancer cells compared to non-tumor cells PNT2.

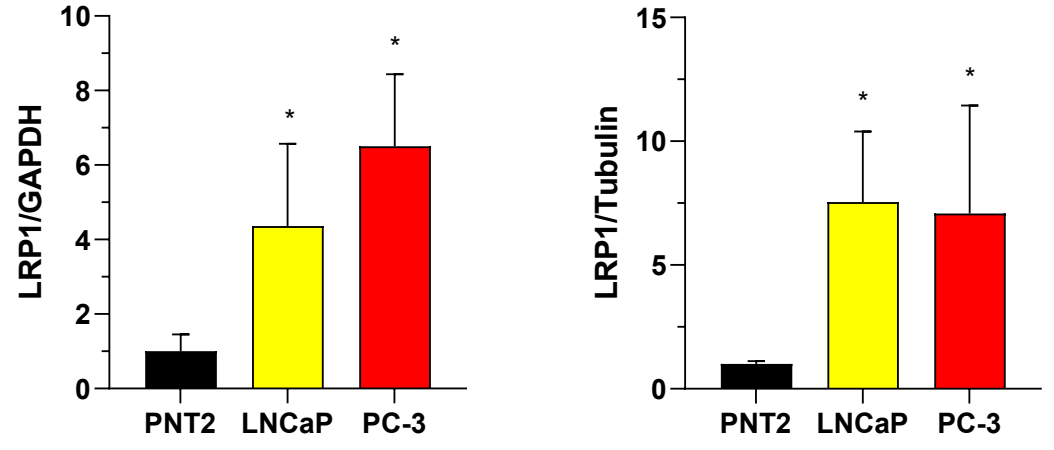


*Figure 23. Gene expression of LRP1 in prostate cancer cells compared to PNT2 cells. Gene expression was analysed by Real-Time PCR in PNT2, LNCaP and PC-3. Protein expression of LRP1 in PNT2, LNCaP and PC-3 cells. Data are expressed as mean±SD, *p<0.05 vs PNT2. Protein expression was analysed via western blot in PNT2, LNCaP and PC-3.*

HMGCoAR

HMG-CoA reductase is the enzyme that limits the rate of synthesis of cholesterol through the mevalonate pathway. Therefore, its gene and protein expression were evaluated in the three cell lines (Figure 24).

The mRNA levels for HMG-CoA reductase in LNCaP and PC3 cells were significantly higher compared to non-tumor cells (approximately 4 times higher, $p<0.05$ LNCaP vs PNT2, $p<0.05$ PC-3 vs PNT2). Protein expression levels of HMGCoA reductase were not significantly different between the three cell lines.

The results obtained show a discrepancy between the gene and protein expression of the enzyme HMGCoA reductase in the LNCaP and PC-3 cell lines.

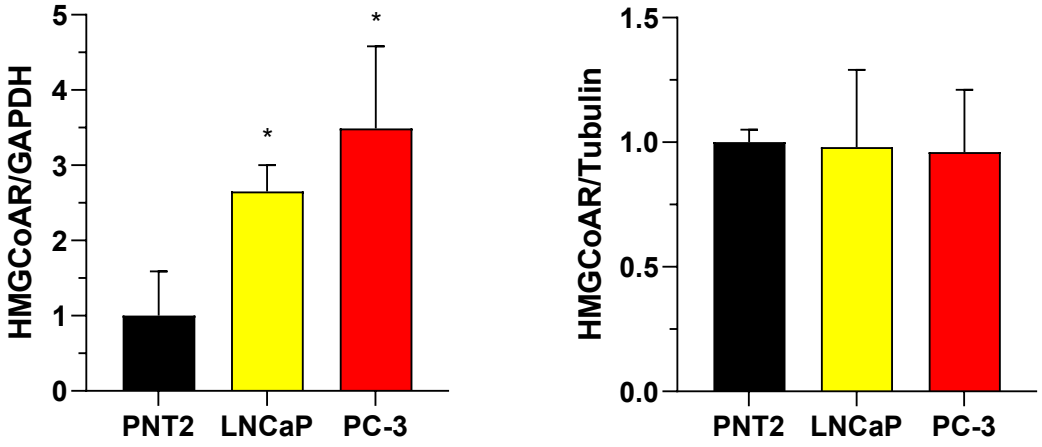


*Figure 24. Gene expression of HMGCoAR in prostate cancer cells compared to PNT2 cells. Gene expression was analysed by Real-Time PCR in PNT2, LNCaP and PC-3. Protein expression of HMGCoAR in PNT2, LNCaP and PC-3 cells. Data are expressed as mean±SD, *p<0.05 vs PNT2. Protein expression was analysed via western blot in PNT2, LNCaP and PC-3.*

SREBP2

Since the transcription factor of the SREBPs family, SREPB2, promotes the synthesis of cholesterol and the endocytosis of lipoproteins, the gene expression of SREBF2 and its regulator SCAP was assessed in the three cell lines (Figure 25).

The mRNA levels of SREBF2 were significantly higher (approximately 4 times higher, $p<0.05$) in both PCa cell lines LNCaP and PC-3 compared to the non-tumor cell PNT2.

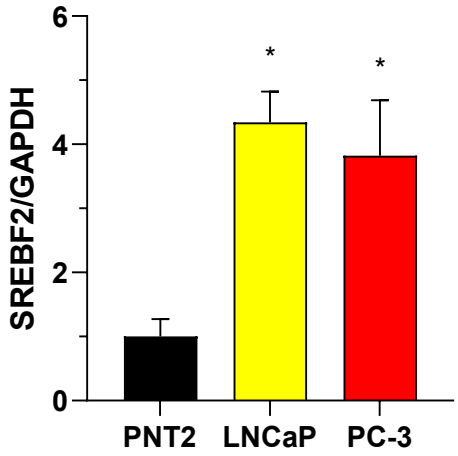


*Figure 25. Gene expression of SREBF2 in prostate cancer cells compared to PNT2 cells. Gene expression was analysed by Real-Time PCR in PNT2, LNCaP and PC-3. Data are expressed as mean±SD, *p<0.05 vs PNT2.*

SRD5A1

The expression of SRD5A1 (steroid 5 alpha-reductase 1), the gene that codes for 5-alpha reductase, that is the enzyme that converts testosterone to dihydrotestosterone, was evaluated in the three cell lines (Figure 26).

The mRNA levels of SRD5A1 in tumor cell lines were significantly higher compared to non-tumor cells. There was a trend increase in mRNA levels proportional to the aggressiveness of the phenotype.

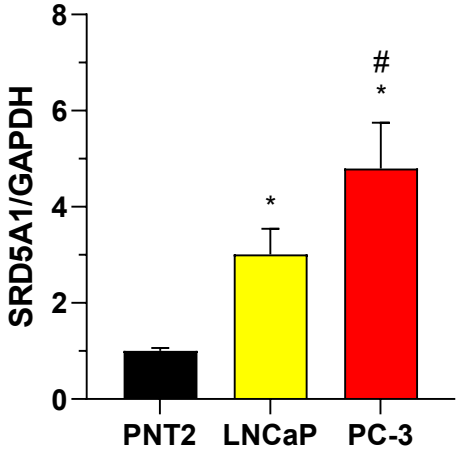


*Figure 26. Gene expression of SRD5A1 in prostate cancer cells compared to PNT2 cells. Gene expression was analysed by Real-Time PCR in PNT2, LNCaP and PC-3. Data are expressed as mean±SD, *p<0.05 vs PNT2, #p<0.05 vs LNCaP.*

Cholesterol content and proliferation induced by LDL loading in PCa cells

To evaluate whether cell cholesterol content in LNCaP was affected by lipoproteins, LNCaP cells were treated with LDL or HDL for 1 hour (Figure 27). The treatment with LDL significantly increased cell cholesterol content (+98%, $p<0.05$) compared to untreated cells. Conversely, the treatment with HDL did not modify cell cholesterol content.

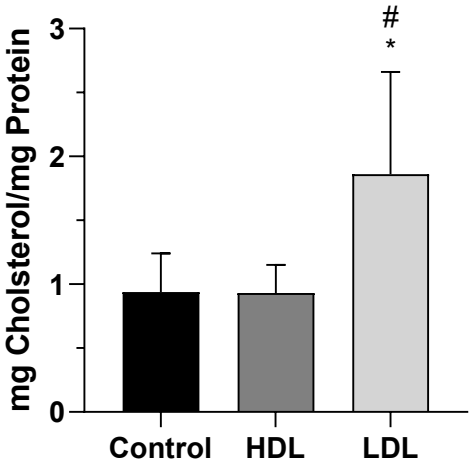


*Figure 27. Cell cholesterol content in LNCaP. Cholesterol levels were measured in fluorescence after 1h of treatment with HDL (0.5 mg/mL) or LDL (50 µg/ml). Data are expressed as content of cholesterol per mg of protein, mean±SD, n=9, #p<0.05 vs CTR, *p<0.05 vs HDL.*

Then, when cells were pre-loaded with cholesterol by o/n incubation with LDL (+52%, $p<0.05$), the subsequent exposure of LNCaP cells to HDL or apoA-I significantly reduced cell cholesterol content (-24%, $p<0.05$) (Figure 28).

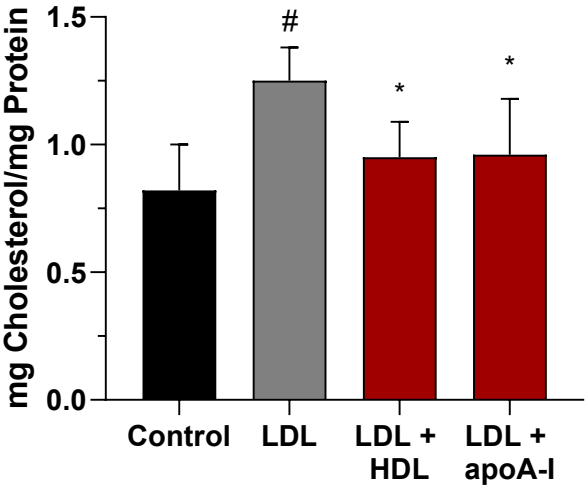


*Figure 28. Cell cholesterol content in LNCaP treated with LDL, HDL or apoA-I. Cells were loaded with LDL (50 µg/ml) and then exposed for 1h to HDL (0.5 mh/mL) or apoA-I (0.5 mg/ml). Data are expressed as cholesterol content per mg of protein, mean±SD, n=9, #p<0.05 vs CTR, *p<0.05 vs LDL.*

Then to evaluate whether the modification of cell cholesterol content could influence cell proliferation, cell number was evaluated in LNCaP. LNCaP cells were incubated with LDL for the first 24 hours, and cell number evaluated after additional 48 hours was increased of 20% ($p<0.05$). However, when cells were treated with HDL for 48 hours, after LDL treatment for 24 hours, there was a reduction of the proliferative effect of 10% ($p<0.05$) compared to cell treated with LDL (Figure 29). MTS assay was used to confirm the data in these cell lines. Thus, indicate that HDL-mediated reduction of cholesterol content inhibits cell proliferation in androgen-dependent LNCaP cells.

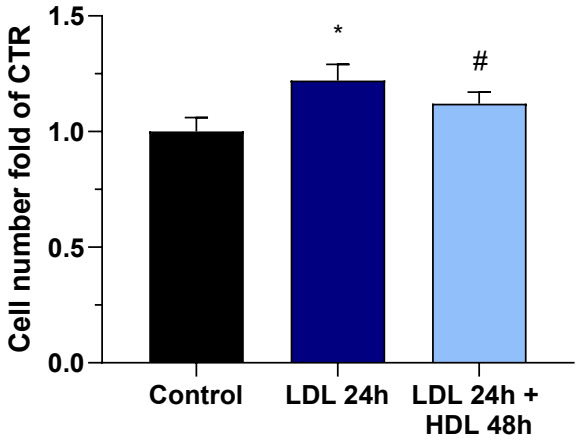


*Figure 29. Effect of LDL and HDL on LNCaP proliferation. Cells were incubated with LDL (50 µg/ml) for 24h and with HDL (0.5 mg / ml) for 48h. They were subsequently collected after 72h and counted through Burker's chamber. Data are expressed as cell number, mean±SD. *p<0.05 vs LDL, #p<0.05 vs CTR.*

The same experiments were performed in castration-resistant PC-3 cells. To evaluate whether cell cholesterol content in these cells was modified by the presence of lipoproteins, PC-3 cells were treated with LDL or HDL for 1 hour (Figure 30).

In PC-3 the treatment not only with LDL (+75%, $p<0,05$) but also with HDL (+51%, $p<0.05$) significantly increased cell cholesterol content compared to untreated cells.

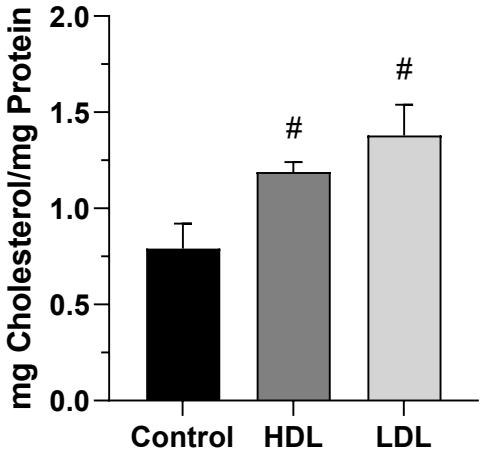


Figure 30. Cell cholesterol content in PC-3. Cholesterol levels were measured in fluorescence after 1h of treatment with HDL (0.5 mg/mL) or LDL (50 µg/ml). Data are expressed as content of cholesterol per mg of protein, mean±SD, n=15, #p<0.05 vs CTR.

Then, cells were pre-loaded with cholesterol by overnight incubation with LDL (+38%, p<0,05), and then treated with HDL or apoA-I that were not able to influence cell cholesterol content (Figure 31).

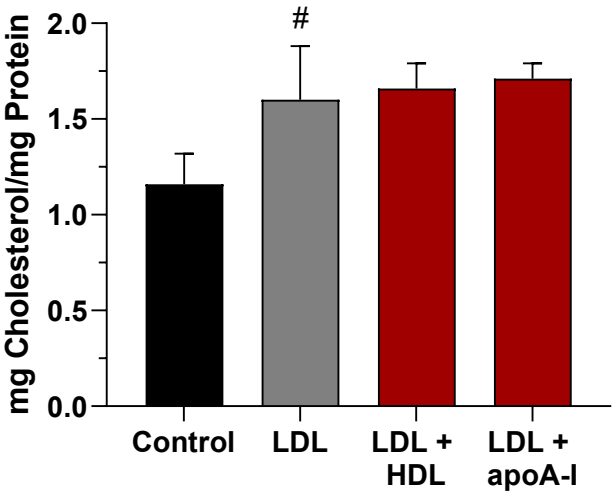


Figure 31. Cell cholesterol content in PC-3 treated with LDL, HDL or apoA-I. Cells were loaded with LDL (50 µg/ml) and then exposed for 1h to HDL (0.5 mh/mL) or apoA-I (0.5 mg/ml). Data are expressed as cholesterol content per mg of protein, mean±SD, n=15, #p <0.05 vs CTR.

Consequently, the treatment with HDL, after 24 hours exposure with LDL (+43%, p<0,05), did not show anti-proliferative effect in PC-3, according to the lack of cell cholesterol reduction (Figure 32). MTS assay was used to confirm the data in these cell lines.

Thus, indicate that HDL lost their ability to reduce cholesterol content and did not inhibit cell proliferation in castration-resistant PC-3 cells.

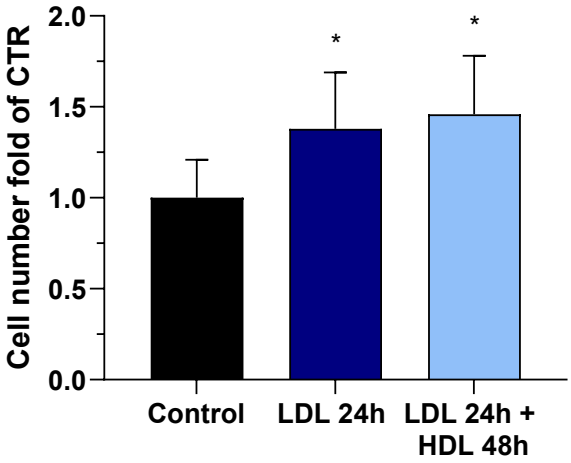


*Figure 32. Effect of LDL and HDL on PC-3 proliferation. Cells were incubated with LDL (50 µg/ml) for 24h and with HDL (0.5 mg / ml) for 48h. They were subsequently collected after 72h and counted through Burker's chamber. Data are expressed as cell number, mean±SD. *p<0.05 vs CTR.*

Role of SR-BI

The possible involvement of SR-BI in cholesterol efflux in PCa cell lines was evaluated by RNA-interference. The treatment with specific siRNAs caused a marked inhibition of SR-BI protein expression; indeed, SR-BI protein expression was reduced by 60% ($p<0.05$) (Figure 33).

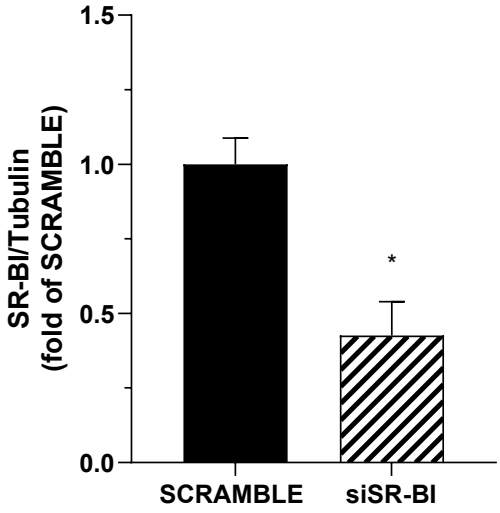


*Figure 33. Protein expression of SR-BI was analysed via western blot in PC-3 cells treated with specific or control (scrambled) siRNA. Data are expressed as mean±SD, *p<0.05 vs SCRAMBLE.*

Then to evaluate whether HDL could impact in the efflux of cholesterol mediated by SR-BI, PCa cells were silenced and treated with HDL for 1 hour, and cell cholesterol content was

evaluated (Figure 34). In LNCaP cells the treatment with HDL cause a significant decrease of cholesterol content in scrambled and in silenced cells compared to untreated cells. In PC-3 cells the treatment with HDL cause a significant increase of cholesterol content in scrambled and in silenced cells compared to untreated cells. However, even when SR-BI was silenced, HDL caused an increase of cell cholesterol content. These results indicate that SR-BI is not involved in this process, strengthening the role of ABCA1 in cholesterol homeostasis of PC-3 cells.

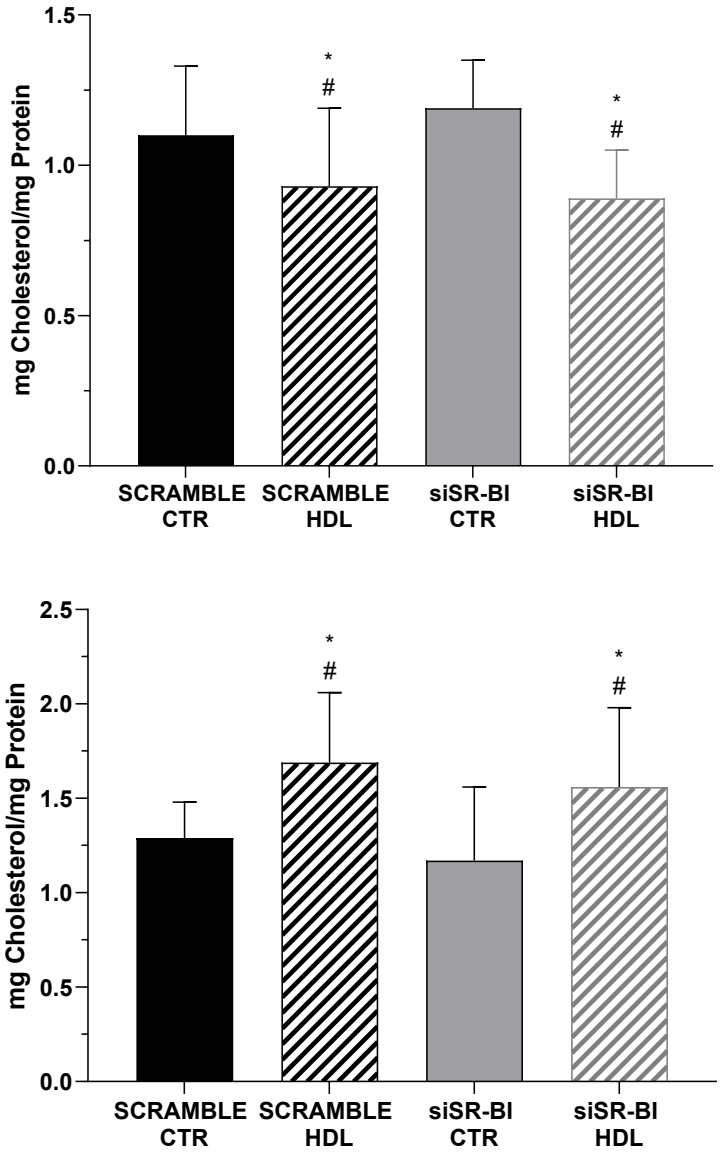


*Figure 34. Cell cholesterol content in LNCaP (Panel A) and PC-3 (Panel B) silenced for SR-BI and treated with HDL. Cells were loaded for 1h with HDL (0.5 mg/ml). Data are expressed as cholesterol content per mg of protein, mean±SD, n=6, *p<0.05 vs SCRAMBLE CTR, #p<0.05 vs siSR-BI CTR.*

Possible causes of cholesterol efflux impairment in PC-3 cells

The possible causes could be the reduction of free cholesterol availability in cell membrane or the modification in terms of expression of receptors and transporters involved in efflux of cholesterol.

Availability of membrane cholesterol

To assess whether the lack of cell cholesterol reduction in PC-3 exposed to HDL was due to a decrease of free cholesterol in cell membrane available for the efflux, PC-3 cells were treated with β-methyl-cyclodextrin (βMCD), a molecule able to promote cell cholesterol efflux through a passive diffusion mechanism. To evaluate the effect of exposure of cancer cells to βMCD on cholesterol content, PC-3 cells were treated o/n with LDL and subsequently were exposed to βMCD (2.5 mM) for 1 hour (Figure 35). Treatment led to a significant reduction in cell cholesterol levels (-24%, $p<0.05$) compared to exposure with LDL alone. This result shows that free cholesterol in membrane is available for the efflux and suggests possible alterations in the transporters and/or receptor that are involved in the efflux of cholesterol in PC-3 cells.

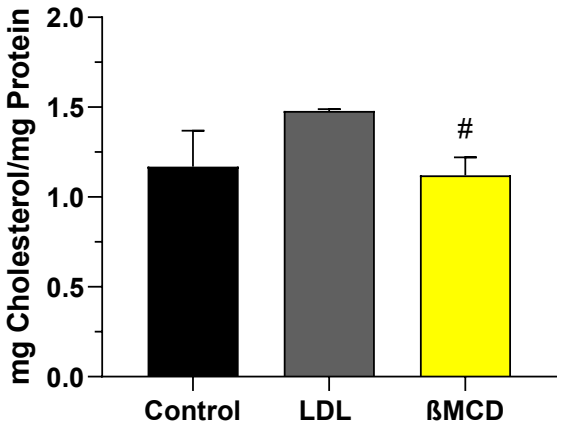


Figure 35. Cell cholesterol content in PC-3 treated with ßMCD. Cells were loaded o/n with LDL (50 µg/ml) and exposed for 1h to ßMCD (2.5 mM). Data are expressed as cholesterol content per mg of protein, mean±SD, n=15, #p<0.05 vs LDL.

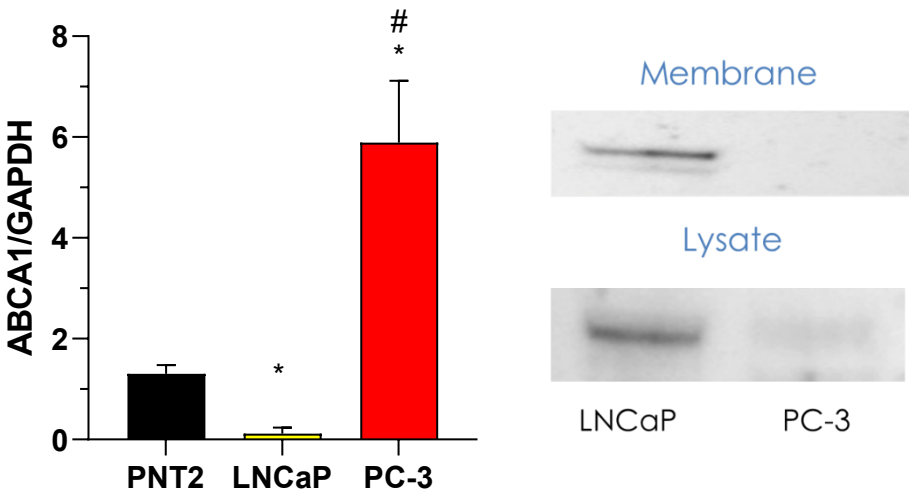
Moreover, unesterified cholesterol to total cholesterol ratio was used to exclude the increased cholesterol esterification in PC-3.

Modification in transporters and receptor involved in cholesterol efflux

Another hypothesis was that transporters and receptor involved in cholesterol efflux are modified in PC-3. The main transporters involved in cholesterol efflux belong to ATP binding cassette family, ABCA1 and ABCG1 mediate an active transport of cholesterol. While the scavenger receptor SR-BI mediates a bidirectional facilitated diffusion of cholesterol. Their expressions were measured by western blot, like shown before (Figure 19).

Role of ABCA1

Since the transporter ABCA1 have a central role in the efflux of cholesterol, its expression was further evaluated. As we have seen, the expression of ABCA1 is relatively lower in cancer cells than in non-tumoral line (-69% in LNCaP and -81% in PC-3). To investigate the expression of ABCA1 in cancer cells, gene expression was measured by Real Time PCR (Figure 36). The mRNA levels of ABCA1 in LNCaP cells was low (-91%) compared to non-tumoral cells PNT2, but the protein was detectable in cell membrane. On the contrary the mRNA levels of ABCA1 in PC-3 cells was markedly increase (4.5 fold increase) compared to PNT2 cells, but low levels of protein was detected in cell lysate and the protein was not present in membrane. The results obtained show a discrepancy between the gene expression and the protein expression of the transporter PC-3 cell line. This suggests that the *ABCA1* gene is transcribed and the protein subsequently degraded. These results could explain the absence of cholesterol efflux in PC-3 cells when they were exposed to HDL or apoA-I.



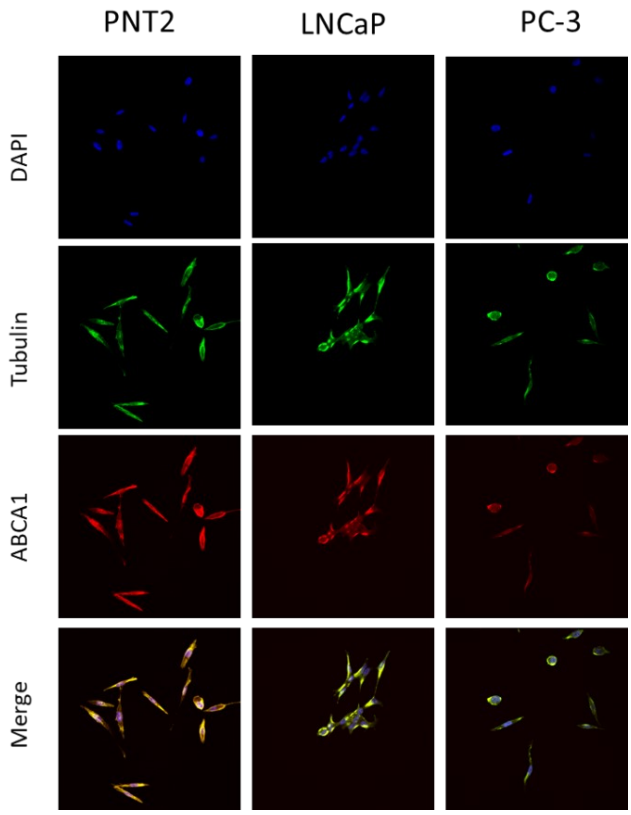


*Figure 36. Gene expression of ABCA1 was analysed by Real-Time PCR in prostate cancer cells compared to PNT2 cells. Protein expression of ABCA1 was analysed via western blot and immunofluorescence in LNCaP and PC-3 cells. Data are expressed as mean±SD, *p<0.05 vs PNT2, #p<0.05 vs PNT2. Fluorescence intensity of ABCA1 in PNT2, LNCaP and PC-3 cells. The images were acquired with confocal microscopy. Nuclei coloured in blue (DAPI), ABCA1 coloured in red and tubulin coloured in green.*

Proteasome inhibition and ABCA1

Thus, in PC-3 cell cholesterol efflux process is altered and in particular the transcription, translation and intracellular transport processes of ABCA1 are modified. The mechanisms responsible for ABCA1 down-regulation was investigated.

By direct sequencing, the presence of mutations in *ABCA1* gene was excluded.

Then the possible involvement of the main processes of protein degradation that could be responsible for the downregulation of ABCA1 was evaluated. To this aim three different compounds inhibitors of the three main degradation pathways was tested: Chloroquine diphosphate that is an inhibitor of lysosomal proteases, Calpeptin that is an inhibitor of calpain protease and MG132 that is an inhibitor of proteasome. The effect on ABCA1 expression was evaluated by immunofluorescence (Figure 37).

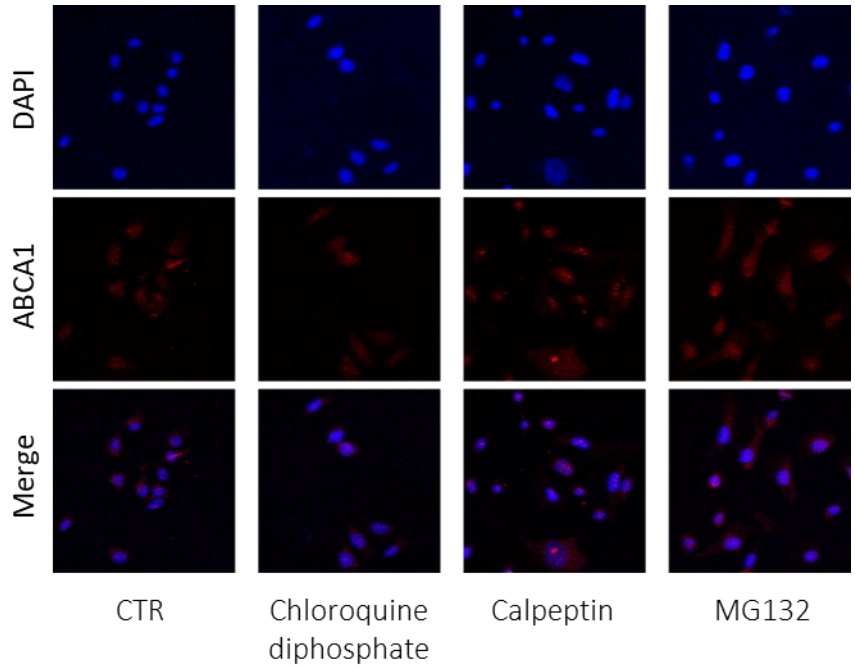


Figure 37. Fluorescence intensity of ABCA1 protein expression in PC-3 cells treated with chloroquine diphosphate (100µM for 8h), calpeptin (30µg/ml for 4h) and MG132 (50µM for 4h) compared with untreated cells. The images were acquired with confocal microscopy. Nuclei coloured in blue (DAPI), ABCA1 coloured in red and tubulin coloured in green.

All treatments led to increased ABCA1 levels, but only the treatment with MG132 showed an increase of 80% of ABCA1 expression compared to control (Figure 38). This result suggested that ABCA1 protein degradation was due to the proteasome.

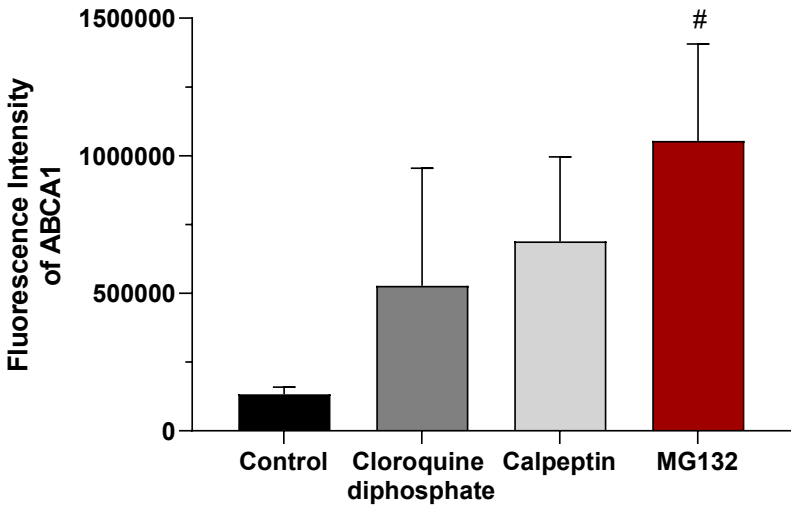


Figure 38. Fluorescence intensity of ABCA1 in PC-3 cells treated with chloroquine diphosphate (100µM for 8h), calpeptin (30µg/ml for 4h) and MG132 (50µM for 4h) compared with untreated cells. Data are expressed as mean±SD, #p<0.05 vs CTR.

To confirm the efficacy of MG132 in the restoration of the expression of ABCA1, and therefore the efflux of cholesterol, cell cholesterol content was assessed in PC-3 cells treated with MG132 and subsequently exposed to HDL for 1 hour (Figure 39).
When PC-3 cells were pre-treated with MG132 for 8 hours, the subsequent exposure to HDL cause a decrease of cell cholesterol content (-28%).

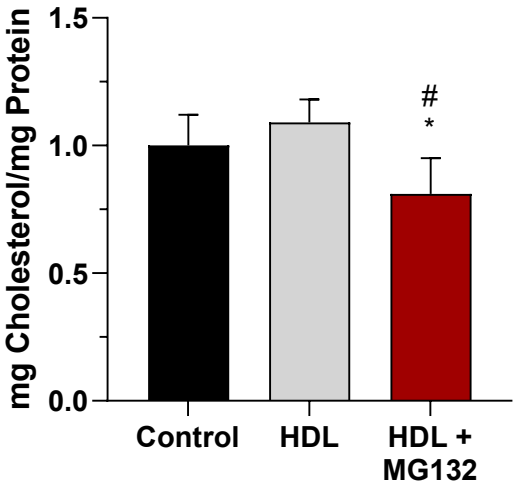


*Figure 39. Cell cholesterol content in PC-3 treated with HDL or co-incubated with MG132. Cells were loaded with HDL for 1h (0.5 mg/ml) and then exposed to MG132 for 8h (50μM). Data are expressed as cholesterol content per mg of protein, mean±SD, n=6, *p <0.05 vs CTR, #p<0.05 vs HDL.*

MG132 is a peptide, which effectively blocks the proteolytic activity of the 26S proteasome complex. The 26S proteasome is a multicatalytic enzyme complex that degrades ubiquitinated proteins via an ATP-dependent mechanism. It is known that ubiquitin-proteasome system has a role in protein homeostasis, that include all that cellular processes involved in the life cycle of proteins, including their synthesis, folding, transport, interactions, and degradation. Dysfunction on this system could contribute to the development of cancer.
One of the proteasome inhibitors was approved by the U.S. FDA as anticancer agents, this is the bortezomib (pirazilcarbonil-Phe-Leu-boronato), that is a boronic acid dipeptide, which acts as a potent and selective 26S proteasome inhibitor.

An immunofluorescence analysis was performed on PC-3 cells treated with bortezomib to evaluate whether bortezomib works on PC-3 cells. PC-3 cells were incubated with or without the inhibitor. The treatment with bortezomib increase of 30% the ABCA1

expression compared to untreated cells (Figure 40). This increase in ABCA1 expression did not affect proliferation.

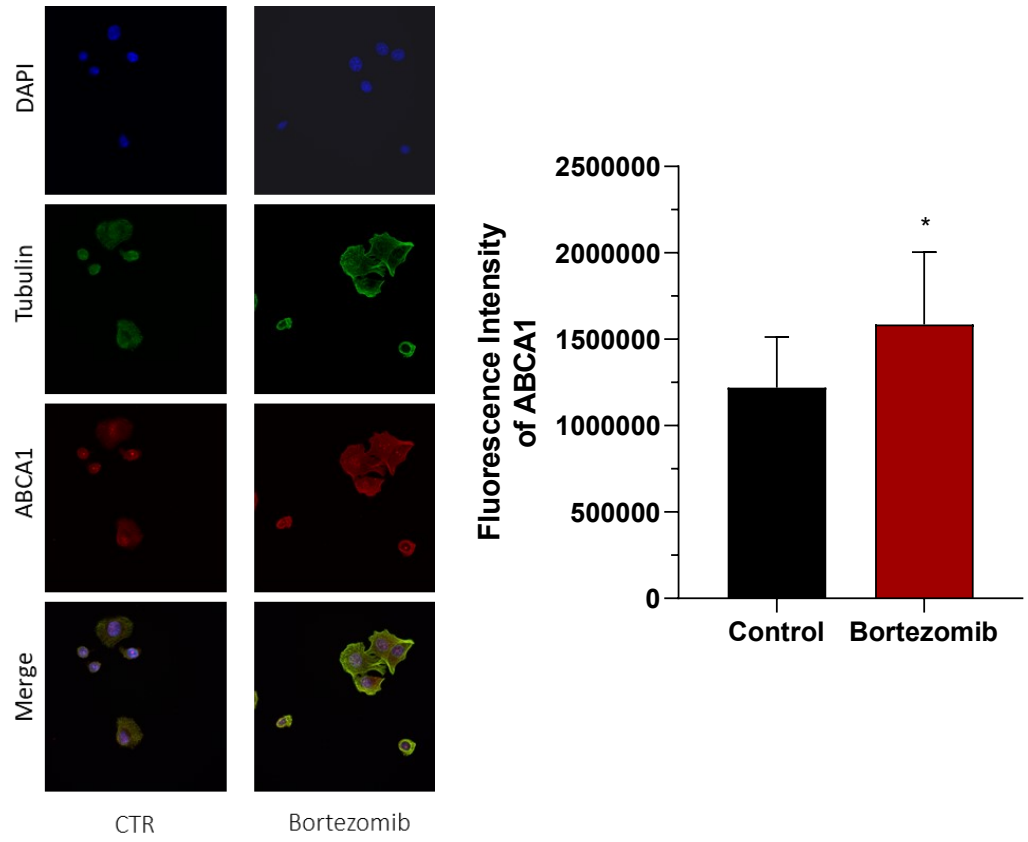


*Figure 40. Fluorescence intensity of ABCA1 protein expression in PC-3 cells treated with bortezomib (5nM for 8h) compared with untreated cells. The images were acquired with confocal microscopy. Nuclei coloured in blue (DAPI), ABCA1 coloured in red and tubulin coloured in green. Data are expressed as mean±SD, *p<0.05 vs CTR.*

Cell cholesterol content and proliferation after treatment with bortezomib

To verify whether the increased expression of ABCA1 can restore the ability of HDL to reduce cell cholesterol, PC-3 were loaded with cholesterol by incubation with LDL and subsequently exposed to HDL with or without pre-treatment with bortezomib (Figure 41). As already demonstrated in previous experiments, LDL treatment causes a significant increase in cell cholesterol content compared to baseline (+72%). Subsequent treatment with HDL, as expected, did not affect the cholesterol content. However, the simultaneous incubation of HDL and bortezomib resulted in a significant decrease in cholesterol compared to cells treated with LDL (-34%) and with LDL and HDL (-33%). These results demonstrate that bortezomib, by inhibiting the proteasome, is able to restore the expression of ABCA1 and consequently allows HDL to reduce the cholesterol content in

androgen-independent cell lines. So, HDL, after the ABCA1 expression is restored, act as cholesterol acceptors.

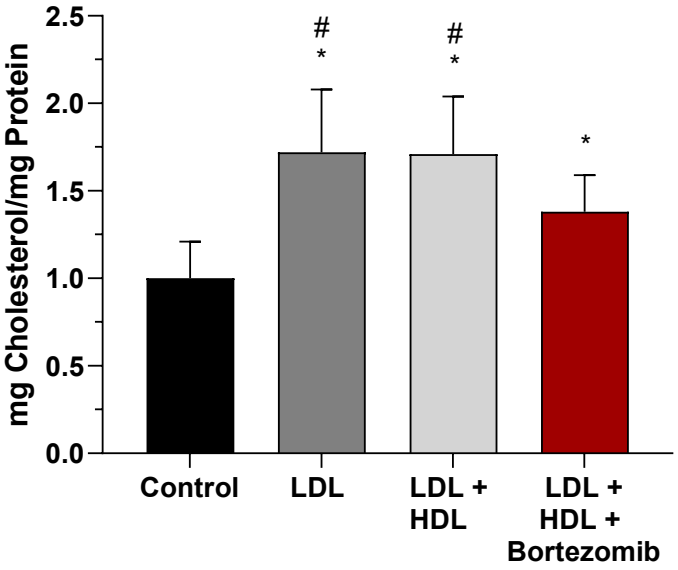


*Figure 41. Cell cholesterol content in PC-3 treated with LDL, HDL or co-incubated with bortezomib. Cells were loaded with LDL (50 µg/ml), HDL (0.50 mg/ml) and then exposed to bortezomib (5 nM). Data are expressed as cholesterol content per mg of protein, mean±SD, n=15, *p<0,05 vs CTR, #p<0,05 vs LDL+HDL+bortezomib.*

The restoration of ABCA1 expression by bortezomib also resulted in a change in cell proliferation (Figure 42). When PC-3 were incubated with LDL for 24 hours, a 20% increase in cell proliferation occurred. As we have seen, HDL alone have not been able to reduce cellular cholesterol content and consequently not even cell proliferation in PC-3 cells.

The concentration of bortezomib used had no direct effect on cell proliferation. In fact the treatment with bortezomib alone in presence of LDL did not influence cell number.

But the subsequent treatment with HDL, after the restoration of ABCA1 expression, reduced cell proliferation by 25% in PC-3 cells.

These results indicate that HDL impact on PC-3 cell proliferation after the restoration of ABCA1 expression in these cells.

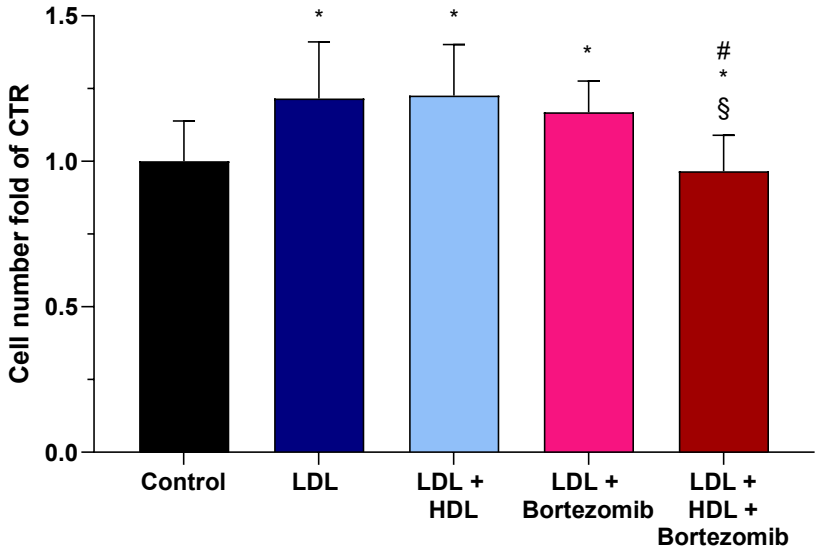


*Figure 42. Effect of LDL and HDL after co-incubation with bortezomib on PC-3 proliferation. Cells were incubated with LDL (50 µg/ml) for 24h, then treated with HDL (0.5 mg / ml) for 48h or bortezomib (5 nM) for 8h, or co-incubated with HDL and bortezomib. They were subsequently collected after 72h and counted through Burker's chamber. Data are expressed as cell number, mean±SD. *p<0,05 vs CTR, #p<0,05 vs LDL, °p<0,05 vs LDL+HDL, §p<0,05 vs LDL+bortezomib.*

Long lasting antiproliferative effect of HDL in vitro

Finally, the antiproliferative effect of HDL has also been investigated with colony formation assay (Figure 43).

The cells were seeded at low density and kept in growth medium in incubation for 14 days after which colony counts containing at least 50 cells were counted. PC-3 cells were treated with HDL for 48 hours and then left to incubate until the end of the two weeks. HDL treatment led to a significant 50% reduction in the number of colonies compared to control. Furthermore, concomitant treatment with HDL and bortezomib reduced the number of colonies by a further 20% compared to treatment with HDL alone. This effect is comparable to the 75% reduction in androgen-dependent LNCaP cells when treated with HDL alone.

These results indicate that the effect of HDL on cancer cells is long-term.

Furthermore, in PC-3 cells HDL were able to inhibit long-term proliferation, although we have seen that HDL were unable to remove cholesterol, this indicates that other mechanisms are involved in inhibiting proliferation, such as antioxidant effect. or anti-inflammatory effect.

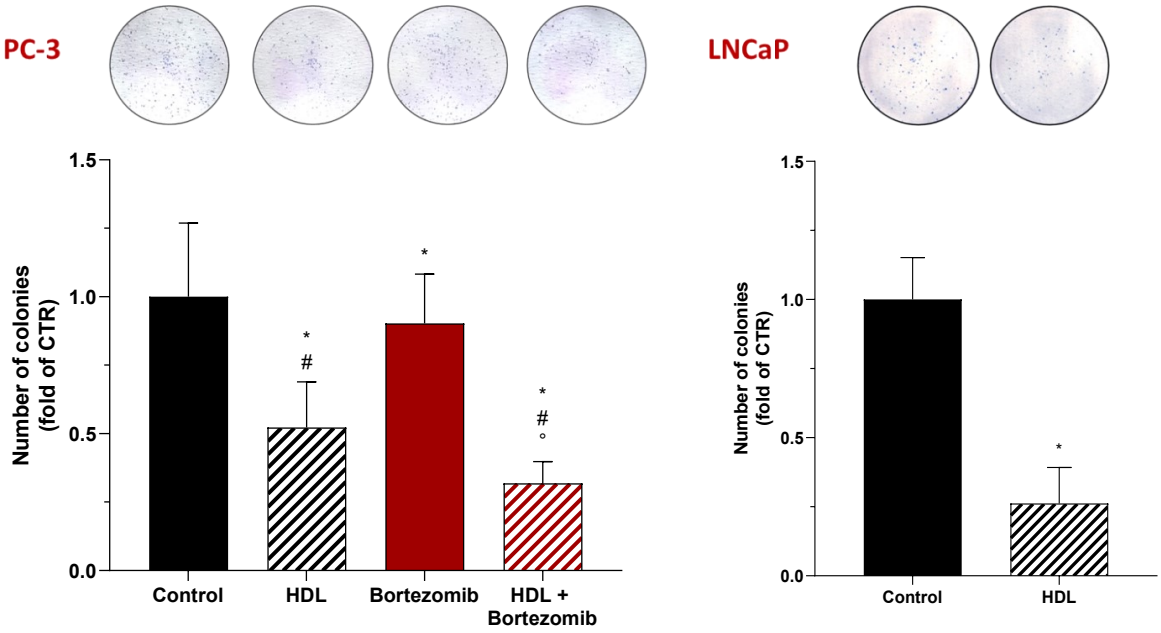


*Figure 43. Effect of HDL and bortezomib on the formation of colonies. Cells were treated with HDL and/or bortezomib (25pM) and after 14 days colonies were counted with optical microscope. Representative images of the colony formation assay experiment. Data are expressed as mean±SD, N=16. *p<0,05 vs CTR, °p<0,05 vs HDL, #p<0,05 vs bortezomib*

Discussion

Data collected within the present project showed that HDL can modulate factors involved in the proliferation of prostate cancer cells, as they can reduce oxidative stress and cell cholesterol content. These processes were shown to be differently modulated depending on the stage of tumor progression, from androgen-dependent to castration-resistant phenotype.

Epidemiological data showed that the incidence of prostate cancer is very high in the male population; therapeutic approaches aimed at androgenic deprivation have good success, but a significant percentage of cases progress towards an androgen-independent form (CRPC), characterized by an unfavorable prognosis (*Lorente et al., 2014*). At now, it is necessary to develop new therapeutic approaches against CRPC or able to interfere with the progression of androgen-dependent tumor to CRPC.

Cancer cells need cholesterol to proliferate, for its structural function in the composition of cell membranes, and as precursor to the synthesis of hormones (*Twiddy et al., 2011*). In addition, different studies show how microenvironment changes can promote tumor expansion and progression. In particular, an increase in oxidative stress and the release of pro-inflammatory molecules have been associated with higher proliferation of prostate cancer cells (*Casey et al., 2015*). In this context, molecules that exert anti-inflammatory and antioxidant activities and modulate cell cholesterol content could have anti-tumor potential. HDL are anti-atherogenic lipoproteins that limit the development of atherosclerotic plaques in the vessel wall thanks to their ability to transport cell cholesterol from peripheral tissues to the liver for its elimination through bile. In addition, they exert antioxidant and anti-inflammatory effects on different cell types involved in the atherosclerotic process (*Calabresi et al., 2010*). These properties, if extended to cancer cells, could highlight for HDL an anti-tumor or adjuvant potential in addition to classic anti-cancer therapy.

This hypothesis was investigated in this project using *in vitro* studies on two cell models of prostate cancer representative of the androgen-dependent (LNCaP) and castration-resistant (PC-3) phenotype, compared with non-tumoral prostate cells (PNT2).

The relevance of oxidative stress to PCa development, progression and conversion to a castration-resistant phenotype has been demonstrated *in vitro* and *in vivo* (*Khandrika et al., 2009*). ROS are generated in cells by mitochondria and by extra mitochondrial enzymatic systems as NADPH oxidases, and in tumor microenvironment by xenobiotics or inflammatory cells (*Paschos et al., 2013*). Interestingly, a lot of experiments highlighted that PC-3 cells, which resemble the more aggressive PCa phenotype, display higher ROS levels than androgen-dependent and non-tumorigenic ones (*Kumar et al., 2008*); in addition, other results showed that short- or long-term exposure to HDL reduced basal oxidative stress in both cell lines. Moreover, the antioxidant potential of HDL was tested in an extreme condition, as an acute challenge with hydrogen peroxide (H_2O_2). Data obtained showed that H_2O_2 was able to significantly increase oxidative stress and proliferation in both cell types. In contrast the treatment with HDL was able to limit H_2O_2-induced oxidative stress and, consequently, oxidative stress-induced proliferation in both PCa cells lines. HDL were effective not only when given in combination with the pro-oxidant stimulus but also when added before it, even for only 1 hour; thus, HDL were able to rapidly modify PCa cells, making them more resistant to the following pro-oxidant stimuli.

It is well known that HDL are able to exert their antioxidant activity, through their ability to uptake oxidized lipids from cell membranes and other lipoproteins, and to carry them to the liver. In addition, proteins carried by HDL can also exert direct antioxidant effect, as for example PON1, LCAT and PAF-A that are well known as antioxidant enzymes; in addition the main component of HDL, apoA-I and apoA-II, are involved in redox reactions through oxidation on methionine residues (*Calabresi et al., 2010*).

Despite of their properties, HDL are not suitable for the development as drugs; they are too heterogeneous and their isolation from human plasma poses key safety concerns. However, HDL-based therapeutic approaches are currently under study. Synthetic HDL made of the main protein and phospholipid components of HDL, namely apoA-I and phosphatidylcholine, were shown to retain most of plasma-derived HDL activities *in vitro* and *in vivo* and are currently in the clinical phase of development as anti-atherosclerotic agents in the context of acute coronary syndrome (*Krause et al., 2013*). These synthetic particles are different from the majority of circulating HDL in plasma. First, they display a discoidal and not a spherical shape resembling the nascent particles called preβ-HDL that

usually account for the 10–15% of total HDL. Second, the protein and lipid composition of synthetic HDL is very simple and, in particular, they do not contain cholesterol, which is not considered responsible for any anti-atherosclerotic effect of HDL (*Calabresi et al., 2006*). An advantage of sHDL use is that they are "customizable", as their shape, size and protein/lipid composition can be modulated to achieve maximal effect.

Data obtained show that sHDL retained the antioxidant capacity of plasma-derived HDL, thus strengthening the idea that sHDL could be investigated as adjuvant anti-tumoral agents. Indeed, when used at the same protein concentration, their antioxidant potential was equal to that of plasma-derived HDL.

Cancer cells need cholesterol to proliferate (*Twiddy et al., 2012*). In fact, cholesterol is an essential component of cell membranes, both structurally and functionally. Gene expression analyses showed that genes and proteins involved in the uptake of cholesterol were over-expressed in cancer cells compared to the non-tumor line.

Both cell models, LNCaP and PC-3, used had a similar cell cholesterol content. Data obtained show that LDL were able to significantly increase cell cholesterol content and induce proliferation in both cell types. However, LNCaP were able to uptake cholesterol only from LDL, while PC-3 increased cell cholesterol content in presence of both LDL and HDL. Subsequently, it was assessed whether the reduction of HDL-mediated cell cholesterol could change the proliferation of the two cancer cell lines. In LNCaP, the exposure to LDL increased cell cholesterol content and consequently also cell proliferation; co-incubation with HDL prevented this proliferative effect probably because of cholesterol reduction. In contrast, in PC-3 there was no decrease in HDL-induced proliferation, accordingly with the non-reduction of cell cholesterol. In addition, HDL and their main component apoA-I protein promoted cholesterol efflux from LNCaP, but not from PC-3.

This absence of efflux was not due to the lack of unesterified cholesterol in membrane, as demonstrated by the reduction of cell cholesterol by passive diffusion in PC-3 treated with βMCD. Moreover, the increased cholesterol esterification in PC-3 was excluded by the unaltered unesterified cholesterol to total cholesterol ratio. The expression of the receptors and transporters involved in the cholesterol efflux process towards HDL and apoA-I in the two cell lines was then assessed. Data obtained through western blot and Real Time PCR shown that in PCa cells there was a high expression in ABCG1 and SR-BI,

which mediate the efflux to mature HDL, while ABCA1 was very poorly expressed compared to non-tumor prostate epithelium cells PNT2.

The SR-BI scavenger receptor was highly expressed in normal prostate epithelium and did not show significant alterations after tumor transformation. The role of SR-BI in cell cholesterol homeostasis is particularly controversial, since it facilitates the bidirectional flux of cholesterol between HDL and cancer cell, depending on gradient concentration (*Menard et al., 2018*).

Since it could be responsible for the intake of cholesterol from HDL in PC-3 cells, SR-BI was silenced by RNA interference. But even when SR-BI was silenced, HDL caused an increase in cell cholesterol content, indicating that SR-BI is not involved in this increase.

Studies in the field of atherosclerosis have shown that the ABCA1 transporter, belonging to the ATP-binding cassette family, is the main responsible for the efflux of cell cholesterol by active transport (*Adorni et al., 2007*). In LNCaP cells, ABCA1 gene and protein expression was significantly reduced compared to the non-tumor line, an effect attributable to the hypermethylation of the promoter of ABCA1, as demonstrated in previous studies (*Lee et al., 2013*). On the contrary, in PC-3 cells, very high levels of mRNA of ABCA1 were detected, but the protein was poorly present in the total cell lysate and almost absent membrane; this could explain the lack of cholesterol efflux from these cells when exposed to HDL or apoA-I.

Then, the mechanisms behind ABCA1 reduced protein expression were investigated, with the hypothesis that by restoring its expression in castration-resistant cell line, HDL could exert the same anti-proliferation action shown in the androgen-dependent line. ABCA1 is a transporter of the ATP-binding family under the transcriptional control of LXR receptors and promotes the efflux of cholesterol and phospholipids to HDL and free apoA-I; its deletion in animals increases the development of atherosclerosis and significantly reduces the biogenesis of HDL at the liver and intestinal level, underlining the central role played in the reverse transport of cholesterol (*Tang et al., 2009*). Moreover, carriers of mutations in the ABCA1 gene display extremely low HDL-C levels and a high cardiovascular risk (*Tang et al., 2009*). Also, in different types of cancer, a reduced expression of ABCA1 has been highlighted, due to both a repression of LXR, and epigenetic modifications such as the hypermethylation of the promoter, as in LNCaP (*Lee et al., 2013*). As protein expression was almost absent in PC-3, despite of the high mRNA levels, a post-transcriptional

alteration was hypothesized. Results showed that inhibition of the proteasome system led to an increase in ABCA1 expression. Proteasome is a highly ordered cellular control system whose main purpose is to eliminate damaged proteins. Proteins, recognized as modified, are bind to ubiquitin, and activate the signal that guides them towards degradation. This system is also involved in the regulation of the cell cycle and apoptosis, so it has been studied as a possible cancer target; for example, proteasome degrades the hypoxia-inducible factor under normoxia (*Bose et al., 2014*). In this study, bortezomib, a small synthetic molecule approved for the treatment of multiple myeloma in humans in 2006 (*Bose et al., 2014*) was used as a proteasome inhibitor. This molecule was used at concentrations that increased the expression of ABCA1 but did not exert a direct anti-proliferation action on PC-3. In PC-3 cells loaded of cholesterol with LDL incubation, the subsequent exposure to HDL after treatment with bortezomib, restored ABCA1 expression and allowed HDL to act as cell cholesterol acceptors, resulting in a reduction in the proliferation of castration-resistant PC-3 cells. The anti-proliferative effect *in vitro* was confirmed using a clonogenic assay. This test showed that a short incubation of PC-3 cells with HDL reduces the number of colonies formed after 14 days, indicating that the anti-proliferative effect of HDL is marked and maintained over time; the simultaneous addition of bortezomib enhanced the effect of HDL, resulting in a further significant decrease in the number of colonies, in the absence of a direct effect of bortezomib to used dose. It should be pointed out that the anti-proliferative effect of HDL in the clonogenic assay cannot be attributed only to the reduction of cellular cholesterol, but it is the result of all the effects that HDL are able to exert on prostate cancer cells, such as the antioxidant action already described.

Finally, ABCG1 protein expression was investigated. It was increased proportionally to the aggressiveness of the phenotype: PC-3 cells shown the highest levels of ABCG1. The relevance of ABCG1 in the efflux of cell cholesterol is still debated, but this transporter has the peculiar ability to promote the movement of oxysterols, products of cholesterol oxidative metabolism (*Yvan-Charvet et al., 2010*). Oxysterols are the endogenous agonists of LXR nuclear receptors, the main regulators of ABCA1 expression. Increased ABCG1 expression could cause an alteration of the cellular content of oxysterols in cancer cells, reducing the activation of the LXR system, which opposes the SREBP system in the context of cholesterol cell homeostasis and is able to exert numerous anti-inflammatory activities

(*Schroepfer, 2000*) (*York et al., 2013*). The effect of the increased ABCG1 expression on the cell content of oxysterols in prostate cancer cells will be the subject of further studies.

Overall, the results obtained in this project support the hypothesis that HDL could affect the proliferation of PCa cells through their known protective mechanisms. From a translational point of view, HDL were effective in androgen-dependent PCa cells, and, after the restoration of ABCA1 expression, they were also effective against castration-resistant ones, for which new effective approaches are strongly needed.

From these results, HDL could have a role as adjuvant agents, able to make cancer cells more sensitive to classic cytotoxic molecules. However, the in vivo relevance of these findings is unknown; a prostate tumor xenograft model will be needed to address this issue and to confirm that HDL are able to affect the proliferation of both androgen-dependent and castration-resistant PCa cells. It could be interesting to inject nude mice with the most aggressive PCa cell lines, treated or not with HDL, and evaluate the development of tumor mass. Moreover, to explore the possibility that the treatment with HDL could make cells more sensitive to classic cytotoxic agents, the in vivo experiment could be repeated with Paclitaxel.

All these results open different ways to identify new therapeutical strategies against castration-resistant prostate cancer.

References

- *Adler, Howard L., et al. "Elevated levels of circulating interleukin-6 and transforming growth factor-beta 1 in patients with metastatic prostatic carcinoma." The Journal of urology 161.1 (1999): 182-187.*
- *Adorni, Maria Pia, et al. "The roles of different pathways in the release of cholesterol from macrophages." Journal of lipid research 48.11 (2007): 2453-2462.*
- *Ahn, Jiyoung, et al. "Prediagnostic total and high-density lipoprotein cholesterol and risk of cancer." Cancer Epidemiology and Prevention Biomarkers 18.11 (2009): 2814-2821.*
- *Alaupovic, Petar, Diana M. Lee, and Walter J. McConathy. "Studies on the composition and structure of plasma lipoproteins: distribution of lipoprotein families in major density classes of normal human plasma lipoproteins." Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 260.4 (1972): 689-707.*
- *Albertsen, Peter C., et al. "Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer." Jama 280.11 (1998): 975-980.*
- *Ambrosi, Glauco, et al. "Anatomia dell'uomo." Seconda Edizione edi-ermes (2001).*
- *Ames, Bruce N. "Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative diseases." Science 221.4617 (1983): 1256-1264.*
- *Apel, Klaus, and Heribert Hirt. "Reactive oxygen species: metabolism, oxidative stress, and signal transduction." Annu. Rev. Plant Biol. 55 (2004): 373-399.*
- *Armandari, Inna, et al. "Intratumoral steroidogenesis in castration-resistant prostate cancer: a target for therapy." Prostate international 2.3 (2014): 105-113.*
- *Ashby, Dale T., et al. "Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells." Arteriosclerosis, thrombosis, and vascular biology 18.9 (1998): 1450-1455.*
- *Åstrand, Ove Alexander Høgmoen, et al. "Synthesis, in vitro and in vivo biological evaluation of new oxysterols as modulators of the liver X receptors." The Journal of steroid biochemistry and molecular biology 165 (2017): 323-330.*
- *Auchus, Mary Louise, and Richard J. Auchus. "Human steroid biosynthesis for the oncologist." Journal of Investigative Medicine 60.2 (2012): 495-503.*
- *Baker, Paul W., et al. "Ability of reconstituted high density lipoproteins to inhibit cytokine-induced expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells." Journal of lipid research 40.2 (1999): 345-353.*
- *Baker, Paul W., et al. "Phospholipid composition of reconstituted high density lipoproteins influences their ability to inhibit endothelial cell adhesion molecule expression." Journal of lipid research 41.8 (2000): 1261-1267.*
- *Barry, Michael J. "Prostate-specific–antigen testing for early diagnosis of prostate cancer." New England Journal of Medicine 344.18 (2001): 1373-1377.*

- *Barter, Philip J., Paul W. Baker, and Kerry-Anne Rye. "Effect of high-density lipoproteins on the expression of adhesion molecules in endothelial cells." Current opinion in lipidology 13.3 (2002): 285-288.*
- *BERTHON, PHILIPPE, et al. "Functional expression of sv40 in normal human prostatic epithelial and fibroblastic cells-differentiation pattern of nontumorigenic cell-lines." International journal of oncology 6.2 (1995): 333-343.*
- *Block, Karen, et al. "Arachidonic Acid–Dependent Activation of a p22phox-Based NAD (P) H Oxidase Mediates Angiotensin II–Induced Mesangial Cell Protein Synthesis and Fibronectin Expression via Akt/PKB." Antioxidants & redox signaling 8.9-10 (2006): 1497-1508.*
- *Bose, Prithviraj, et al. "Bortezomib for the treatment of non-Hodgkin's lymphoma." Expert opinion on pharmacotherapy 15.16 (2014): 2443-2459.*
- *Bovenga, Fabiola, Carlo Sabbà, and Antonio Moschetta. "Uncoupling nuclear receptor LXR and cholesterol metabolism in cancer." Cell metabolism 21.4 (2015): 517-526.*
- *Brar, Sukhdev S., et al. "NOX5 NAD (P) H oxidase regulates growth and apoptosis in DU 145 prostate cancer cells." American Journal of Physiology-Cell Physiology 285.2 (2003): C353-C369.*
- *Braschi, Sylvie, et al. "Apolipoprotein AI charge and conformation regulate the clearance of reconstituted high density lipoprotein in vivo." Journal of lipid research 40.3 (1999): 522-532.*
- *Brewer Jr, H. B., et al. "The amino acid sequence of human APOA-I, an apolipoprotein isolated from high density lipoproteins." Biochemical and biophysical research communications 80.3 (1978): 623-630.*
- *Bruchovsky, N., et al. "Hormonal effects on cell proliferation in rat prostate." Vitamins & Hormones. Vol. 33. Academic Press, 1976. 61-102.*
- *Brunham, Liam R., et al. "Intestinal ABCA1 directly contributes to HDL biogenesis in vivo." The Journal of clinical investigation 116.4 (2006): 1052-1062.*
- *Calabresi, L., Gomaraschi, M., Rossoni, G. & Franceschini, G. Synthetic high density lipoproteins for the treatment of myocardial ischemia/reperfusion injury. Pharmacol Ther 111, 836–854 (2006)*
- *Calabresi, Laura, and Guido Franceschini. "Lecithin: cholesterol acyltransferase, high-density lipoproteins, and atheroprotection in humans." Trends in cardiovascular medicine 20.2 (2010): 50-53.*
- *Calabresi, Laura, et al. "A novel homozygous mutation in CETP gene as a cause of CETP deficiency in a Caucasian kindred." Atherosclerosis 205.2 (2009): 506-511.*
- *Calabresi, Laura, Monica Gomaraschi, and Guido Franceschini. "Endothelial protection by high-density lipoproteins: from bench to bedside." Arteriosclerosis, thrombosis, and vascular biology 23.10 (2003): 1724-1731.*
- *Carter, D. C., and J. X. Ho. "Structure of serum albumin in Advances in Protein Chemistry: Lipoproteins, Apolipoproteins, and Lipases." (1994).*
- *Caruso, Maria Gabriella, et al. "3-Hydroxy-3-methylglutaryl coenzyme A reductase activity and low-density lipoprotein receptor expression in diffuse-type and intestinal-type human gastric cancer." Journal of gastroenterology 37.7 (2002): 504-508.*

- *Casey, Stephanie C., et al. "Cancer prevention and therapy through the modulation of the tumor microenvironment." Seminars in cancer biology. Vol. 35. Academic Press, 2015.*
- *Castelli, William P., et al. "Incidence of coronary heart disease and lipoprotein cholesterol levels: the Framingham Study." Jama 256.20 (1986): 2835-2838.*
- *Celotti, Fabio. Patologia generale e fisiopatologia. EdiSes srl, 2006.*
- *Chang, Qing, et al. "The IL-6/JAK/Stat3 feed-forward loop drives tumorigenesis and metastasis." Neoplasia (New York, NY) 15.7 (2013): 848.*
- *Chapman, Dennis. "Phase transitions and fluidity characteristics of lipids and cell membranes." Quarterly reviews of biophysics 8.2 (1975): 185-235.*
- *Cheng, Liang, et al. "Staging of prostate cancer." Histopathology 60.1 (2012): 87-117.*
- *Cheung, Marian C., and J. J. Albers. "Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing AI and A-II and particles containing AI but no A-II." Journal of Biological Chemistry 259.19 (1984): 12201-12209.*
- *Chisholm, Jeffrey W., et al. "ApoA-I secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA-I and intracellularly assembled nascent HDL." Journal of lipid research 43.1 (2002): 36-44.*
- *Chung, Theodore DK, et al. "Characterization of the role of IL-6 in the progression of prostate cancer." The Prostate 38.3 (1999): 199-207.*
- *Clark, Robert A. "Activation of the neutrophil respiratory burst oxidase." The Journal of infectious diseases 179.Supplement_2 (1999): S309-S317.*
- *Clay, M. A., et al. "Cholesteryl ester transfer protein and hepatic lipase activity promote shedding of apo AI from HDL and subsequent formation of discoidal HDL." Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 1124.1 (1992): 52-58.*
- *Cockerill, Gillian W., et al. "High-density lipoproteins differentially modulate cytokine-induced expression of E-selectin and cyclooxygenase-2." Arteriosclerosis, thrombosis, and vascular biology 19.4 (1999): 910-917.*
- *Coward, Jermaine, et al. "Interleukin-6 as a therapeutic target in human ovarian cancer." Clinical cancer research 17.18 (2011): 6083-6096.*
- *Crawford, E. David, et al. "A controlled trial of leuprolide with and without flutamide in prostatic carcinoma." New England Journal of Medicine 321.7 (1989): 419-424.*
- *Dakubo, G. D., et al. "Altered metabolism and mitochondrial genome in prostate cancer." Journal of clinical pathology 59.1 (2006): 10-16.*
- *Danilo, Christiane, and Philippe G. Frank. "Cholesterol and breast cancer development." Current opinion in pharmacology 12.6 (2012): 677-682.*
- *Davidson, W. Sean, et al. "Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function." Arteriosclerosis, thrombosis, and vascular biology 29.6 (2009): 870-876.*

- *De Beer MC, Durbin DM, Cai L, et al. Apolipoprotein A-II modulates the binding and selective uptake of reconstituted HDL by scavenger receptor BI. J Biol Chem.(2001) 276:15832-9.*
- *Deckelbaum, Richard J., et al. "Abnormal high density lipoproteins of abetalipoproteinemia: relevance to normal HDL metabolism." Journal of lipid research 23.9 (1982): 1274-1282.*
- *Ditiatkovski, Michael, et al. "An apolipoprotein AI mimetic peptide designed with a reductionist approach stimulates reverse cholesterol transport and reduces atherosclerosis in mice." PLoS One 8.7 (2013).*
- *Dixon, Joseph L., and Henry N. Ginsberg. "Hepatic synthesis of lipoproteins and apolipoproteins." Seminars in liver disease. Vol. 12. No. 04. © 1992 by Thieme Medical Publishers, Inc., 1992.*
- *Dufour, Julie, et al. "Oxysterol receptors, AKT and prostate cancer." Current opinion in pharmacology 12.6 (2012): 724-728.*
- *Eckhardt, Erik RM, et al. "High density lipoprotein uptake by scavenger receptor SR-BII." Journal of Biological Chemistry 279.14 (2004): 14372-14381.*
- *Epstein, Jonathan I. "An update of the Gleason grading system." The Journal of urology 183.2 (2010): 433-440.*
- *Favari, Elda, et al. "Probucol inhibits ABCA1-mediated cellular lipid efflux." Arteriosclerosis, thrombosis, and vascular biology 24.12 (2004): 2345-2350.*
- *Favari, Elda, et al. "Small discoidal pre-β1 HDL particles are efficient acceptors of cell cholesterol via ABCA1 and ABCG1." Biochemistry 48.46 (2009): 11067-11074.*
- *Fleisher, Lloyd N., et al. "Stimulation of arterial endothelial cell prostacyclin synthesis by high density lipoproteins." Journal of Biological Chemistry 257.12 (1982): 6653-6655.*
- *Fox, Emily M., Josefa Andrade, and Margaret A. Shupnik. "Novel actions of estrogen to promote proliferation: integration of cytoplasmic and nuclear pathways." Steroids 74.7 (2009): 622-627.*
- *Franceschini, G., L. Calabresi, and A. Corsini. "Controllo farmacologico della lesione aterosclerotica: terapia delle dislipidemie." (2006): 253-267.*
- *Ganapathy, Ekambaram, et al. "D-4F, an apoA-I mimetic peptide, inhibits proliferation and tumorigenicity of epithelial ovarian cancer cells by upregulating the antioxidant enzyme MnSOD." International journal of cancer 130.5 (2012): 1071-1081.*
- *Gao, Feng, et al. "Apolipoprotein AI mimetic peptides inhibit expression and activity of hypoxia-inducible factor-1α in human ovarian cancer cell lines and a mouse ovarian cancer model." Journal of Pharmacology and Experimental Therapeutics 342.2 (2012): 255-262.*
- *Gao, Feng, et al. "L-5F, an apolipoprotein AI mimetic, inhibits tumor angiogenesis by suppressing VEGF/basic FGF signaling pathways." Integrative Biology 3.4 (2011): 479-489.*
- *Gao, Lina, et al. "Androgen receptor promotes ligand-independent prostate cancer progression through c-Myc upregulation." PloS one 8.5 (2013).*

- *Garber, D. W., Datta, G., Chaddha, M., Palgunachari, M. N., Hama, S. Y., Navab, M., ... & Anantharamaiah, G. M. (2001). A new synthetic class A amphipathic peptide analogue protects mice from diet-induced atherosclerosis. Journal of lipid research, 42(4), 545-552.*
- *Getz, Godfrey S., and Catherine A. Reardon. "Apolipoprotein AI and AI mimetic peptides: a role in atherosclerosis." Journal of inflammation research 4 (2011): 83.*
- *Gomaraschi, Monica, et al. "Anti-inflammatory and cardioprotective activities of synthetic high-density lipoprotein containing apolipoprotein AI mimetic peptides." Journal of Pharmacology and Experimental Therapeutics 324.2 (2008): 776-783.*
- *Gomaraschi, Monica, et al. "CLINICAL PERSPECTIVE." Circulation 116.19 (2007): 2165-2172.*
- *Gomaraschi, Monica. "Role of Lipoproteins in the Microenvironment of Hormone-Dependent Cancers." Trends in Endocrinology & Metabolism (2019).*
- *Gordon, Tavia, et al. "High density lipoprotein as a protective factor against coronary heart disease: the Framingham Study." The American journal of medicine 62.5 (1977): 707-714.*
- *Goyal, Jaya, et al. "Novel Function of Lecithin-Cholesterol Acyltransferase HYDROLYSIS OF OXIDIZED POLAR PHOSPHOLIPIDS GENERATED DURING LIPOPROTEIN OXIDATION." Journal of Biological Chemistry 272.26 (1997): 16231-16239.*
- *Graff, Julie N., and Tomasz M. Beer. "Should docetaxel be administered earlier in prostate cancer therapy?." (2015): 977-979.*
- *Gregory, Christopher W., et al. "A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy." Cancer research 61.11 (2001): 4315-4319.*
- *Gu, Feifei, et al. "Structures of discoidal high density lipoproteins a combined computational-experimental approach." Journal of Biological Chemistry 285.7 (2010): 4652-4665.*
- *Halliwell, Barry. "Reactive oxygen species in living systems: source, biochemistry, and role in human disease." The American journal of medicine 91.3 (1991): S14-S22.*
- *Hamid, Agus Rizal AH, et al. "Aldo-keto reductase family 1 member C3 (AKR1C3) is a biomarker and therapeutic target for castration-resistant prostate cancer." Molecular Medicine 18.11 (2012): 1449-1455.*
- *Heemers, Hannelore V., and Donald J. Tindall. "Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex." Endocrine reviews 28.7 (2007): 778-808.*
- *Heinecke, Jay W. "The HDL proteome: a marker–and perhaps mediator–of coronary artery disease." Journal of lipid research 50.Supplement (2009): S167-S171.*
- *Heinonen, Olli P., et al. "Prostate cancer and supplementation with α-tocopherol and β-carotene: incidence and mortality in a controlled trial." JNCI: Journal of the National Cancer Institute 90.6 (1998): 440-446.*
- *Hodge, Kathryn K., et al. "Random systematic versus directed ultrasound guided transrectal core biopsies of the prostate." The Journal of urology 142.1 (1989): 71-74.*

- *Horton, J. D., J. L. Goldstein, and M. S. Brown. "SREBPs: transcriptional mediators of lipid homeostasis." Cold Spring Harbor symposia on quantitative biology. Vol. 67. Cold Spring Harbor Laboratory Press, 2002.*
- *Hricak, Hedvig, and Peter T. Scardino. "Current clinical issues in prostate cancer that can be addressed by imaging." This page intentionally left blank (2009): 29.*
- *Huuskonen, Jarkko, et al. "The impact of phospholipid transfer protein (PLTP) on HDL metabolism." Atherosclerosis 155.2 (2001): 269-281.*
- *Ikewaki, K., et al. "Evaluation of apoA-I kinetics in humans using simultaneous endogenous stable isotope and exogenous radiotracer methods." Journal of lipid research 34.12 (1993): 2207-2215.*
- *Jafri, Haseeb, Alawi A. Alsheikh-Ali, and Richard H. Karas. "Baseline and on-treatment high-density lipoprotein cholesterol and the risk of cancer in randomized controlled trials of lipid-altering therapy." Journal of the American College of Cardiology 55.25 (2010): 2846-2854.*
- *Jahangiri, A., et al. "Evidence that endothelial lipase remodels high density lipoproteins without mediating the dissociation of apolipoprotein AI." Journal of lipid research 46.5 (2005): 896-903.*
- *Jauhiainen, M., K. J. Stevenson, and P. J. Dolphin. "Human plasma lecithin-cholesterol acyltransferase. The vicinal nature of cysteine 31 and cysteine 184 in the catalytic site." Journal of Biological Chemistry 263.14 (1988): 6525-6533.*
- *Jessup, Wendy, et al. "Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages." Current opinion in lipidology 17.3 (2006): 247-257.*
- *Kenny, Paraic A., Genee Y. Lee, and Mina J. Bissell. "Targeting the tumor microenvironment." Frontiers in bioscience: a journal and virtual library 12 (2007): 3468.*
- *Khandrika, L., Kumar, B., Koul, S., Maroni, P., & Koul, H. K. Oxidative stress in prostate cancer. 282, 125–136 (2009).*
- *Khandrika, Lakshmipathi, et al. "Oxidative stress in prostate cancer." Cancer letters 282.2 (2009): 125-136.*
- *Kim, Yong-Wan, et al. "Development of multiplexed bead-based immunoassays for the detection of early stage ovarian cancer using a combination of serum biomarkers." PloS one 7.9 (2012).*
- *Kiss, Robert S., et al. "The lipidation by hepatocytes of human apolipoprotein AI occurs by both ABCA1-dependent and-independent pathways." Journal of Biological Chemistry 278.12 (2003): 10119-10127.*
- *Kloudova, Alzbeta, F. Peter Guengerich, and Pavel Soucek. "The role of oxysterols in human cancer." Trends in Endocrinology & Metabolism 28.7 (2017): 485-496.*
- *Koivisto, Pasi, et al. "Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer." Cancer research 57.2 (1997): 314-319.*
- *Kozak, Katherine R., et al. "Characterization of serum biomarkers for detection of early stage ovarian cancer." Proteomics 5.17 (2005): 4589-4596.*
- *Kozyraki, Renata, et al. "The human intrinsic factor-vitamin B12 receptor, cubilin: molecular characterization and chromosomal mapping of the gene to 10p within the autosomal recessive*

megaloblastic anemia (MGA1) region." Blood, The Journal of the American Society of Hematology 91.10 (1998): 3593-3600.

- *Krause, B. R. & Remaley, A. T. Reconstituted HDL for the acute treatment of acute coronary syndrome. Curr Opin Lipidol 24, 480–486 (2013).*
- *Kumar, B., Koul, S., Khandrika, L., Meacham, R. B. & Koul, H. K. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. Cancer Res 68, 1777–1785 (2008).*
- *Kumar, Binod, et al. "Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype." Cancer research 68.6 (2008): 1777-1785.*
- *Kunitake, S. T., et al. "Pre-beta high density lipoprotein. Unique disposition of apolipoprotein AI increases susceptibility to proteolysis." Arteriosclerosis: An Official Journal of the American Heart Association, Inc. 10.1 (1990): 25-30.*
- *Kuvin, Jeffrey T., et al. "A novel mechanism for the beneficial vascular effects of high-density lipoprotein cholesterol: enhanced vasorelaxation and increased endothelial nitric oxide synthase expression." American heart journal 144.1 (2002): 165-172.*
- *Lee, Byron H., et al. "Dysregulation of cholesterol homeostasis in human prostate cancer through loss of ABCA1." Cancer research 73.3 (2013): 1211-1218.*
- *Lemon, Bryan, and Robert Tjian. "Orchestrated response: a symphony of transcription factors for gene control." Genes & development 14.20 (2000): 2551-2569.*
- *Lewis, Daniel R., et al. "Polymer-based therapeutics: nanoassemblies and nanoparticles for management of atherosclerosis." Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 3.4 (2011): 400-420.*
- *Li, Xiang-An, et al. "High density lipoprotein binding to scavenger receptor, Class B, type I activates endothelial nitric-oxide synthase in a ceramide-dependent manner." Journal of Biological Chemistry 277.13 (2002): 11058-11063.*
- *Liao, Guoqing, et al. "Regulation of androgen receptor activity by the nuclear receptor corepressor SMRT." Journal of Biological Chemistry 278.7 (2003): 5052-5061.*
- *Lim, So Dug, et al. "Increased Nox1 and hydrogen peroxide in prostate cancer." The Prostate 62.2 (2005): 200-207.*
- *Lin, Ching-Yu, et al. "Cholestane-3β, 5α, 6β-triol suppresses proliferation, migration, and invasion of human prostate cancer cells." PloS one 8.6 (2013).*
- *Lopez, Alan D., et al. "Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data." The lancet 367.9524 (2006): 1747-1757.*
- *Lorente, D., and J. S. De Bono. "Molecular alterations and emerging targets in castration resistant prostate cancer." European journal of cancer 50.4 (2014): 753-764.*
- *Lund-Katz, Sissel, and Michael C. Phillips. "High density lipoprotein structure–function and role in reverse cholesterol transport." Cholesterol Binding and Cholesterol Transport Proteins:. Springer, Dordrecht, 2010. 183-227.*

- *Mackness, Michael I., Paul N. Durrington, and Bharti Mackness. "How high-density lipoprotein protects against the effects of lipid peroxidation." Current opinion in lipidology 11.4 (2000): 383-388.*
- *Marathe, Gopal K., Guy A. Zimmerman, and Thomas M. McIntyre. "Platelet-activating factor acetylhydrolase, and not paraoxonase-1, is the oxidized phospholipid hydrolase of high density lipoprotein particles." Journal of Biological Chemistry 278.6 (2003): 3937-3947.*
- *Marks, Leonard S., Elahe A. Mostaghel, and Peter S. Nelson. "Prostate tissue androgens: history and current clinical relevance." Urology 72.2 (2008): 247-254.*
- *Martinez, Laurent O., et al. "Ectopic β-chain of ATP synthase is an apolipoprotein AI receptor in hepatic HDL endocytosis." Nature 421.6918 (2003): 75-79.*
- *McDonnell, Donald P., et al. "Obesity, cholesterol metabolism, and breast cancer pathogenesis." Cancer research 74.18 (2014): 4976-4982.*
- *McGrath, K. C. Y., et al. "Role of 3β-hydroxysteroid-Δ24 reductase in mediating antiinflammatory effects of high-density lipoproteins in endothelial cells." Arteriosclerosis, thrombosis, and vascular biology 29.6 (2009): 877-882.*
- *McNeal, John E. "Anatomy of the prostate: an historical survey of divergent views." The prostate 1.1 (1980): 3-13.*
- *Menard, Julien A., Myriam Cerezo-Magaña, and Mattias Belting. "Functional role of extracellular vesicles and lipoproteins in the tumour microenvironment." Philosophical Transactions of the Royal Society B: Biological Sciences 373.1737 (2018): 20160480.*
- *Mineo, Chieko, et al. "High density lipoprotein-induced endothelial nitric-oxide synthase activation is mediated by Akt and MAP kinases." Journal of Biological Chemistry 278.11 (2003): 9142-9149.*
- *Mishra, V. K., et al. "Interactions of synthetic peptide analogs of the class A amphipathic helix with lipids. Evidence for the snorkel hypothesis." Journal of Biological Chemistry 269.10 (1994): 7185-7191.*
- *Moestrup, Søren K., and Lars Bo Nielsen. "The role of the kidney in lipid metabolism." Current opinion in lipidology 16.3 (2005): 301-306.*
- *Moestrup, Søren K., and Renata Kozyraki. "Cubilin, a high-density lipoprotein receptor." Current opinion in lipidology 11.2 (2000): 133-140.*
- *Montgomery, R. Bruce, et al. "Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth." Cancer research 68.11 (2008): 4447-4454.*
- *Morishita, Hiroshi, et al. "Increased hydrolysis of cholesteryl ester with prostacyclin is potentiated by high density lipoprotein through the prostacyclin stabilization." The Journal of clinical investigation 86.6 (1990): 1885-1891.*
- *Morita, Shin-ya, et al. "Pluronic L81 affects the lipid particle sizes and apolipoprotein B conformation." Chemistry and physics of lipids 126.1 (2003): 39-48.*
- *Morrissey, Colm, et al. "The expression of osteoclastogenesis-associated factors and osteoblast response to osteolytic prostate cancer cells." The Prostate 70.4 (2010): 412-424.*
- *Mostaghel, Elahe A., et al. "Impact of circulating cholesterol levels on growth and intratumoral androgen concentration of prostate tumors." PloS one 7.1 (2012).*

- *Movva, Rajesh, and Daniel J. Rader. "Laboratory assessment of HDL heterogeneity and function." Clinical Chemistry 54.5 (2008): 788-800.*
- *Naber, Kurt G., and Wolfgang Weidner. "Chronic prostatitis—an infectious disease?." Journal of Antimicrobial Chemotherapy 46.2 (2000): 157-161.*
- *Naugler, Willscott E., and Michael Karin. "The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer." Trends in molecular medicine 14.3 (2008): 109-119.*
- *Navab, Mohamad, et al. "HDL and the inflammatory response induced by LDL-derived oxidized phospholipids." Arteriosclerosis, thrombosis, and vascular biology 21.4 (2001): 481-488.*
- *Navab, Mohamad, et al. "Structure and function of HDL mimetics." Arteriosclerosis, thrombosis, and vascular biology 30.2 (2010): 164-168.*
- *Navab, Mohamad, et al. "Thematic review series: the pathogenesis of atherosclerosis the oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL." Journal of lipid research 45.6 (2004): 993-1007.*
- *Nelson, David L., and Michael M. Cox. Lehninger principles of biochemistry. New York: WH Freeman, 2009.*
- *Nelson, William G., et al. "The role of inflammation in the pathogenesis of prostate cancer." The Journal of urology 172.5 (2004): S6-S12.*
- *Newmark, Jay R., et al. "Androgen receptor gene mutations in human prostate cancer." Proceedings of the National Academy of Sciences 89.14 (1992): 6319-6323.*
- *Nguyen, Daniel P., Jinyi Li, and Ashutosh K. Tewari. "Inflammation and prostate cancer: the role of interleukin 6 (IL-6)." BJU international 113.6 (2014): 986-992.*
- *Nicholls, Stephen J., et al. "Relationship between atheroma regression and change in lumen size after infusion of apolipoprotein AI Milano." Journal of the American College of Cardiology 47.5 (2006): 992-997.*
- *Nigon, Fabienne, et al. "Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor." Journal of lipid research 32.11 (1991): 1741-1753.*
- *Ohvo-Rekilä, Henna, et al. "Cholesterol interactions with phospholipids in membranes." Progress in lipid research 41.1 (2002): 66-97.*
- *Olinski, Ryszard, et al. "Oxidative DNA damage: assessment of the role in carcinogenesis, atherosclerosis, and acquired immunodeficiency syndrome." Free Radical Biology and Medicine 33.2 (2002): 192-200.*
- *Paschos, A., Pandya, R., Duivenvoorden, W. C. & Pinthus, J. H. Oxidative stress in prostate cancer: changing research concepts towards a novel paradigm for prevention and therapeutics. Prostate Cancer Prostatic Dis 16, 217–225 (2013).*
- *Pelton, Kristine, Michael R. Freeman, and Keith R. Solomon. "Cholesterol and prostate cancer." Current opinion in pharmacology 12.6 (2012): 751-759.*

- *Petrylak, Daniel P., et al. "Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer." New England Journal of Medicine 351.15 (2004): 1513-1520.*
- *Phillips, Michael C. "Molecular mechanisms of cellular cholesterol efflux." Journal of Biological Chemistry 289.35 (2014): 24020-24029.*
- *Pisani, Enrico, et al. "Patologie prostatiche."*
- *Polascik, Thomas J., Joseph E. Oesterling, and Alan W. Partin. "Prostate specific antigen: a decade of discovery-what we have learned and where we are going." The Journal of urology 162.2 (1999): 293-306.*
- *Pomerantz, Kenneth B., et al. "Enrichment of endothelial cell arachidonate by lipid transfer from high density lipoproteins: relationship to prostaglandin I2 synthesis." Journal of lipid research 26.10 (1985): 1269-1276.*
- *Rader, Daniel J. "Molecular regulation of HDL metabolism and function: implications for novel therapies." The Journal of clinical investigation 116.12 (2006): 3090-3100.*
- *Raggers, René J., et al. "Lipid traffic: the ABC of transbilayer movement." Traffic 1.3 (2000): 226-234.*
- *Rallidis, Loukianos S., and John Lekakis. "PCSK9 inhibition as an emerging lipid lowering therapy: Unanswered questions." Hellenic Journal of Cardiology 57.2 (2016): 86-91.*
- *Rämet, Maria E., et al. "High-density lipoprotein increases the abundance of eNOS protein in human vascular endothelial cells by increasing its half-life." Journal of the American College of Cardiology 41.12 (2003): 2288-2297.*
- *Raut, Sangram, et al. "Reconstituted HDL: drug delivery platform for overcoming biological barriers to cancer therapy." Frontiers in pharmacology 9 (2018): 1154.*
- *Raza, Shaneabbas, et al. "The cholesterol metabolite 27-hydroxycholesterol stimulates cell proliferation via ERß in prostate cancer cells." Cancer cell international 17.1 (2017): 52.*
- *Repa, Joyce J., and David J. Mangelsdorf. "The role of orphan nuclear receptors in the regulation of cholesterol homeostasis." Annual review of cell and developmental biology 16.1 (2000): 459-481.*
- *Ricci, Francesco, et al. "Safety of antiandrogen therapy for treating prostate cancer." Expert opinion on drug safety 13.11 (2014): 1483-1499.*
- *Rigotti, Attilio, et al. "A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism." Proceedings of the National Academy of Sciences 94.23 (1997): 12610-12615.*
- *Rigotti, Attilio, et al. "Scavenger receptor BI--a cell surface receptor for high density lipoprotein." Current opinion in lipidology 8.3 (1997): 181-188.*
- *Rocco, Alessandro Guerini, et al. "Structural features and dynamics properties of human apolipoprotein AI in a model of synthetic HDL." Journal of Molecular Graphics and Modelling 28.4 (2009): 305-312.*
- *Roheim, Paul S. "INTRODUCTION TO LIPOPROTEIN METABOLISM, APOLIPOPROTEINS, LIPID CONSTITUENTS." Nutrition, Digestion, Metabolism. Pergamon, 1981. 487-488.*

- *Rosenson, Robert S., et al. "Fenofibrate reduces fasting and postprandial inflammatory responses among hypertriglyceridemia patients with the metabolic syndrome." Atherosclerosis 198.2 (2008): 381-388.*
- *Ross, Russell, and John A. Glomset. "Atherosclerosis and the arterial smooth muscle cell." Science 180.4093 (1973): 1332-1339.*
- *Rothblat, George H., and Michael C. Phillips. "High-density lipoprotein heterogeneity and function in reverse cholesterol transport." Current opinion in lipidology 21.3 (2010): 229.*
- *Rye, Kerry-Anne, and Philip J. Barter. "Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein AI." Arteriosclerosis, thrombosis, and vascular biology 24.3 (2004): 421-428.*
- *Santoni, Riccardo, et al. "Linee guida carcinoma della prostata-AIRO, 2016." Tumori 2016 (2016): S1-S79.*
- *Saraon, Punit, Keith Jarvi, and Eleftherios P. Diamandis. "Molecular alterations during progression of prostate cancer to androgen independence." Clinical chemistry 57.10 (2011): 1366-1375.*
- *Schaefer, Ernst J., et al. "Human apolipoprotein AI and A-II metabolism." Journal of lipid research 23.6 (1982): 850-862.*
- *Schaefer, Ernst J., et al. "The composition and metabolism of high density lipoprotein subfractions." Lipids 14.5 (1979): 511-522.*
- *Schroepfer Jr, George J. "Oxysterols: modulators of cholesterol metabolism and other processes." Physiological reviews 80.1 (2000): 361-554.*
- *Schwartz, Charles C., Julie M. VandenBroek, and Patricia S. Cooper. "Lipoprotein cholesteryl ester production, transfer, and output in vivo in humans." Journal of lipid research 45.9 (2004): 1594-1607.*
- *Shiao, Stephen L., et al. "Immune microenvironments in solid tumors: new targets for therapy." Genes & development 25.24 (2011): 2559-2572.*
- *Shiota, Masaki, et al. "Hsp27 regulates epithelial mesenchymal transition, metastasis, and circulating tumor cells in prostate cancer." Cancer research 73.10 (2013): 3109-3119.*
- *Silvente-Poirot, Sandrine, Florence Dalenc, and Marc Poirot. "The effects of cholesterol-derived oncometabolites on nuclear receptor function in cancer." Cancer research 78.17 (2018): 4803-4808.*
- *Silver, J. S., and C. A. Hunter. "gp130 at the nexus of inflammation, autoimmunity, and cancer." Journal of leukocyte biology 88.6 (2010): 1145-1156.*
- *Simons, Kai, and Elina Ikonen. "How cells handle cholesterol." Science 290.5497 (2000): 1721-1726.*
- *Sirtori, Cesare R., Laura Calabresi, and Guido Franceschini. "Recombinant apolipoproteins for the treatment of vascular diseases." Atherosclerosis 142.1 (1999): 29-40.*
- *Smith, Jeffrey R., et al. "Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search." Science 274.5291 (1996): 1371-1374.*
- *Sobel, R. E., and M. D. Sadar. "Cell lines used in prostate cancer research: a compendium of old and new lines—part 1." The Journal of urology 173.2 (2005): 342-359.*

- *Sobel, R. E., and M. D. Sadar. "Cell lines used in prostate cancer research: a compendium of old and new lines—part 2." The Journal of urology 173.2 (2005): 360-372.*
- *Spieker, Lukas E., et al. "High-density lipoprotein restores endothelial function in hypercholesterolemic men." Circulation 105.12 (2002): 1399-1402.*
- *Stocker, Roland, and John F. Keaney Jr. "Role of oxidative modifications in atherosclerosis." Physiological reviews 84.4 (2004): 1381-1478.*
- *Su, Feng, et al. "Apolipoprotein AI (apoA-I) and apoA-I mimetic peptides inhibit tumor development in a mouse model of ovarian cancer." Proceedings of the National Academy of Sciences 107.46 (2010): 19997-20002.*
- *Sutcliffe, Siobhan, et al. "Sexually transmitted infections and prostatic inflammation/cell damage as measured by serum prostate specific antigen concentration." The Journal of urology 175.5 (2006): 1937-1942.*
- *Tabet, Fatiha, et al. "The 5A apolipoprotein AI mimetic peptide displays antiinflammatory and antioxidant properties in vivo and in vitro." Arteriosclerosis, thrombosis, and vascular biology 30.2 (2010): 246-252.*
- *Tall, Alan R., et al. "1999 George Lyman Duff memorial lecture: lipid transfer proteins, HDL metabolism, and atherogenesis." Arteriosclerosis, thrombosis, and vascular biology 20.5 (2000): 1185-1188.*
- *Tang, Chongren, and John F. Oram. "The cell cholesterol exporter ABCA1 as a protector from cardiovascular disease and diabetes." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1791.7 (2009): 563-572.*
- *Tannock, Ian F., et al. "Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer." New England Journal of Medicine 351.15 (2004): 1502-1512.*
- *Taplin, Mary-Ellen, et al. "Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer." New England Journal of Medicine 332.21 (1995): 1393-1398.*
- *Terasaka, Naoki, et al. "ABCG1 and HDL protect against endothelial dysfunction in mice fed a high-cholesterol diet." The Journal of clinical investigation 118.11 (2008): 3701-3713.*
- *Timmins, Jenelle M., et al. "Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I." The Journal of clinical investigation 115.5 (2005): 1333-1342.*
- *Twiddy, Alexis L., Carlos G. Leon, and Kishor M. Wasan. "Cholesterol as a potential target for castration-resistant prostate cancer." Pharmaceutical research 28.3 (2011): 423-437.*
- *van der Sluis, Tim M., et al. "Intraprostatic testosterone and dihydrotestosterone. Part I: concentrations and methods of determination in men with benign prostatic hyperplasia and prostate cancer." BJU international 109.2 (2012): 176-182.*
- *Vinals, Marisa, José Martínez-González, and Lina Badimon. "Regulatory effects of HDL on smooth muscle cell prostacyclin release." Arteriosclerosis, thrombosis, and vascular biology 19.10 (1999): 2405-2411.*

- *Vohl, Marie-Claude, et al. "A novel lecithin-cholesterol acyltransferase antioxidant activity prevents the formation of oxidized lipids during lipoprotein oxidation." Biochemistry 38.19 (1999): 5976-5981.*
- *Wang, Nan, et al. "Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in transgenic mice." Journal of Biological Chemistry 273.49 (1998): 32920-32926.*
- *Warburg, Otto. "On the origin of cancer cells." Science 123.3191 (1956): 309-314.*
- *Webb, Nancy R., et al. "Remodeling of HDL remnants generated by scavenger receptor class B type I." Journal of lipid research 45.9 (2004): 1666-1673.*
- *Weisgraber, Karl H., and Robert W. Mahley. "Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography." Journal of lipid research 21.3 (1980): 316-325.*
- *Wu, Chun-Te, et al. "Significance of IL-6 in the transition of hormone-resistant prostate cancer and the induction of myeloid-derived suppressor cells." Journal of molecular medicine 90.11 (2012): 1343-1355.*
- *Xia, Pu, et al. "High density lipoproteins (HDL) interrupt the sphingosine kinase signaling pathway a possible mechanism for protection against atherosclerosis by HDL." Journal of Biological Chemistry 274.46 (1999): 33143-33147.*
- *York, Autumn G., and Steven J. Bensinger. "Subverting sterols: rerouting an oxysterol-signaling pathway to promote tumor growth." Journal of Experimental Medicine 210.9 (2013): 1653-1656.*
- *Young, Robert Henry. Tumors of the prostate gland, seminal vesicles, male urethra, and penis. Amer Registry of Pathology, 2000.*
- *Yu, Liqing, et al. "Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion." Proceedings of the National Academy of Sciences 99.25 (2002): 16237-16242.*
- *Yuhanna, Ivan S., et al. "High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase." Nature medicine 7.7 (2001): 853-857.*
- *Yvan-Charvet, Laurent, Nan Wang, and Alan R. Tall. "Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses." Arteriosclerosis, thrombosis, and vascular biology 30.2 (2010): 139-143.*
- *Zamanian-Daryoush, Maryam, et al. "The cardioprotective protein apolipoprotein A1 promotes potent anti-tumorigenic effects." Journal of Biological Chemistry 288.29 (2013): 21237-21252.*
- *Zechner, Rudolf, et al. "In vitro formation of HDL-2 from HDL-3 and triacylglycerol-rich lipoproteins by the action of lecithin: cholesterol acyltransferase and cholesterol ester transfer protein." Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 918.1 (1987): 27-35.*
- *Zerrad-Saadi, Amal, et al. "HDL3-mediated inactivation of LDL-associated phospholipid hydroperoxides is determined by the redox status of apolipoprotein AI and HDL particle surface lipid rigidity: relevance to inflammation and atherogenesis." Arteriosclerosis, thrombosis, and vascular biology 29.12 (2009): 2169-2175.*
- *Zhang, Zhen, et al. "Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer." Cancer research 64.16 (2004): 5882-5890.*

- *Zhu, Meng-Lei, and Natasha Kyprianou. "Androgen receptor and growth factor signaling cross-talk in prostate cancer cells." Endocrine-related cancer 15.4 (2008): 841.*

Career

- ***Awards and scholarships:***
  - ✓ Scholarship of the Telethon Foundation (project GGP14125)
  - ✓ SIF grant for participation at the Summer School "Cancer biology and therapeutic strategies towards personalized medicine"
  - ✓ SIF scholarship for short abroad period

- ***Courses:***
  - ✓ Introductory course on animal experimentation, Istituto Mario Negri, Milano
  - ✓ Prevention of chemical and biological risks in the laboratory (AwareLab), Milano
  - ✓ International Atherosclerosis Society, HDL workshop 2019, Valencia

- ***Congress participations:***
  - ✓ "Next Step VIII, la giovane ricerca avanza" Giornata della Ricerca del Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano - Oral presentation
  - ✓ Giornata della Ricerca del Centro E. Grossi Paoletti, ASST Ospedale Niguarda, Milano – Poster presentation
  - ✓ Convegno della Società Italiana per lo Studio dell'Arteriosclerosi (SISA), sezione regionale Lombardia, Milano - Oral presentation
  - ✓ "Novità e updates sulla prevenzione e cura della malattia cardiovascolare" Incontro tra giovani ricercatori SIIA, SIMI e SISA - Spring Meeting, Rimini – Oral presentation
  - ✓ "New perspectives in pharmacology: from genetic to real life" II Edition Spring School - Chiesa in Valmalenco – Oral presentation
  - ✓ "Cancer biology and therapeutic strategies towards personalized medicine" International Summer School, II Edition – Lipari – Poster presentation
  - ✓ "Next Step IX, la giovane ricerca avanza" Giornata della Ricerca del Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano - Oral presentation
  - ✓ Convegno della Società Italiana per lo Studio dell'Arteriosclerosi (SISA), sezione regionale Lombardia, Milano - Oral presentation
  - ✓ "Novità e updates sulla prevenzione e cura della malattia cardiovascolare" Incontro tra giovani ricercatori SIIA, SIMI e SISA - Spring Meeting, Rimini – Poster presentation
  - ✓ III Edition Spring School - Chiesa in Valmalenco - Oral presentation
  - ✓ "Novità e updates sulla prevenzione e cura della malattia cardiovascolare" Incontro tra giovani ricercatori SIIA, SIMI e SISA - Spring Meeting, Rimini – Poster presentation
  - ✓ IV Edition Spring School - Chiesa in Valmalenco - Oral presentation

- ***Publications:***
  1. LEPTIN AND RESISTIN AFFECT PCSK9 EXPRESSION VIA STAT3 INVOLVEMENT - Macchi C, Botta M, Garzone M, Marchianò S, Giorgio E, Corsini A, Magni P, Ferri N, Ruscica M - Atherosclerosis 2017; 263:e70-e71
  2. LYSOSOMAL ACID LIPASE ACTIVITY IS REDUCED IN NAFLD: MECHANISMS AND RESCUE BY PPAR-ALPHA AGONIST - Gomaraschi M, Francazani A, Pavanello C, Giorgio E, Calabresi L, Fargion S. - Atherosclerosis 2018; 275:e24-25
  3. HIGH DENSITY LIPOPROTEINS INHIBIT OXIDATIVE STRESS-INDUCED PROSTATE CANCER CELL PROLIFERATION - Ruscica M, Botta M, Ferri N, Giorgio E, Macchi C, Franceschini G, Magni P, Calabresi L, Gomaraschi M. - Scientific Reports 2018; 8:2236
  4. DYSFUNCTIONAL HDL AS A THERAPEUTIC TARGET FOR ATHEROSCLEROSIS PREVENTION - Ossoli A, Pavanello C, Giorgio E, Calabresi L, Gomaraschi M. - Current Medicinal Chemistry 2018; Volume 25
  5. TREATEMENT WITH FIBRATES IS ASSOCIATED WITH HIGHER LAL ACTIVITY IN DYSLIPIDEMIC PATIENTS - Pavanello C, Baragetti A, Branchi A, Grigore L, Castelnuovo S, Giorgio E, Catapano A L, Calabresi L, Gomaraschi M. - Pharmacological Research, 2019;147:104362.
  6. LIPID ACCUMULATION IMPAIRS LYSOSOMAL ACID LIPASE ACTIVITY IN HEPATOCYTES: EVIDENCE IN NAFLD PATIENTS AND CELL CULTURES - Gomaraschi M, Fracanzani A, Dongiovanni P, Pavanello C, Giorgio E, Da Dalt L, Norata GD,Calabresi L, Consonni D, Lombardi R, Branchi A, Fargion S. -Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1864.12 (2019): 158523.
  7. LIPA GENE MUTATIONS AFFECT THE COMPOSITION OF LIPOPROTEINS: ENRICHMENT IN ACAT-DERIVED CHOLESTERYL ESTERS - Arnaboldi L, Ossoli A, Giorgio E, Pisciotta L, Lucchi T, Grigore L, Pavanello C, Granata A, Pasta A, Arosio B, Azzolino D, Baragetti A, Castelnuovo S, Corsini A, Catapano AL, Calabresi L, Gomaraschi M - Atherosclerosis. 2020 Mar;297:8-15.