Universidade de Lisboa

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The Role of Systemic Metabolism in Breast Cancer Progression

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Doutoramento em Medicina

Especialidade de Cirurgia Geral

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"Depois de escalar uma grande colina,
apenas descobrimos que há muitas mais colinas para escalar".
Nelson Mandela

Esta frase tem estado bem presente para mim. Aplica-se a cada momento em que trabalhei neste projecto. O trabalho não fica terminado quando concluímos uma tarefa. Isso apenas nos indica que há muitos mais caminhos.

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Resumo

Em cada dez novos casos de cancro diagnosticados, no mundo, por ano, um é cancro de mama. Apesar do aumento da sobrevivência nos países mais desenvolvidos (e com alta incidência), relacionado com a existência de rastreio, diagnóstico rápido e melhoria no tratamento, o cancro da mama continua a ser a principal causa de morte por cancro em mulheres.

A investigação das últimas décadas tem-se centrado fundamentalmente no tumor e na sua genómina considerando-o como uma entidade independente do organismo. Mesmo sendo a terapêutica individualizada um dos objectivos major desta pesquisa, as características do individuo têm sido poucas poucas vezes valorizadas.

A hipótese principal deste trabalho foi a de que o tumor é um microsistema a evoluir num macrosistema hospedeiro devendo existir uma interdependência entre estes. O conceito da influência do macroambiente na evolução tumoral é consistente com a teoria Darwiniana da evolução que postula que a seleção é uma propriedade essencial dos sistemas biológicos. Isto é, assim como as forças ambientais poderão explicar os padrões de incidência do cancro da mama na população mundial, também os factores do hospedeiro poderão selecionar os fenótipos do tumor. De facto, dados recentes indicam que alguns factores de risco conhecidos para o cancro da mama são condições sistémicas como a obesidade ou a diabetes. A prevalência epidémica da obesidade e das suas comorbilidades têm sido investigadas como possíveis razões para o aumento da incidência do cancro nas sociedades ocidentais. No entanto, apesar de as alterações do perfil lipídico, nomeadamente a hipercolesterolémia, serem também prevalentes nestas regiões o papel do metabolismo lipídico sistémico no cancro da mama permanece por esclarecer.

Os lípidos são fundamentais para as células, onde actuam quer como constituintes celulares quer como moléculas de sinalização. O colesterol, especificamente, é um componente essencial das membranas celulares onde se concentra nos denominados “lipid rafts”, microdomínios da membrana envolvidos na polaridade da célula, migração, proliferação, sobrevivência e angiogénese. O colesterol é também o precursor obrigatório das hormonas esteróides como o estrogénio e progesterona, sendo a vasta maioria dos tumores de mama hormono-sensíveis.

Até agora,a maioria dos estudos publicados sobre relação entre o colesterol e o cancro da mama tentaram demonstrar causalidade entre os níveis séricos e a incidência de cancro. Raros estudos exploraram a eventual associação com a agressividade tumoral ou com a sua progressão. Portanto, a importância do colesterol plasmático na progressão do cancro da mama é desconhecida. **Esta Tese visou estudar o papel do colesterol sistémico na progressão do cancro da mama e os mecanismos moleculares subjacentes**

Para tal foi desenhado um estudo observacional prospectivo que seguiu um coorte de mulheres com cancro de mama (estadios I-III), sem tratamento prévio. Os resultados mostraram que o nível de LDL-C no momento do diagnóstico se correlaciona positivamente com o tamanho e estadio do tumor. Aos 2 anos de seguimento, mulheres com níveis de LDL-C mais elevados no diagnóstico tiveram menor tempo livre de doença. Estes dados apontam o LDL-C como um biomarcador de agressividade tumoral (factor de prognóstico) e um possível alvo terapêutico (factor preditivo).

Para provar a relação causal do ambiente enriquecido em colesterol na progressão do tumor foram usados modelos bem estabelecidos *in vitro* e *in vivo* que permitiram demonstrar que a sinalização mediada pelo LDL-C induz fenótipos tumorais, semelhantes aos observados nos doentes com cancro de mama e caracterizados por maior proliferação e capacidade invasora. As células expostas a concentrações maiores de LDL-C apresentam características genotípicas distintas, compatíveis com um fenótipo mais agressivo, como confirmado por análise de expressão génica. Experimentalmente foi demonstrado que a expressão tumoral do ABCA1 (principal exportador celular de cholesterol) num ambiente enriquecido em colesterol é um marcador do fenótipo induzido pelo LDL-C.

Em conjunto, os resultados desta tese ilustram a influência do metabolismo sistémico na progressão do cancro da mama e sugerem que o perfil lipídico deve ser avaliado em todos os doentes com este diagnóstico. Sendo a hipercolesterolémia tão prevalente nas sociedades ocidentais com uma distribuição global paralela à incidência do cancro da mama, é de esperar que a modulação do perfil lipídico nestes doentes tenha um impacto elevado como estratégia de prevenção (secundária). Isto parece particularmente relevante para os doentes com tumores não hormono-sensíveis já que não existem outras formas de quimioprevenção.

Palavras-chave: cancro da mama; colesterol, LDL-C

Abstract

One in ten of all new cancers diagnosed worldwide, each year, is a breast cancer. Despite the more favorable survival of breast cancer patients in (high-incidence) developed regions due to screening, faster diagnosis and improved treatment, it remains the most frequent cause of cancer-related death in women.

The research of the last decades focused primarily on tumor genomics considering the tumor as an entity independent of the organism. Although the personalized therapy has been the ultimate goal of this aproach, host characteristics were seldom valued.

Our working hypothesis states that the tumor is a microsystem evolving within a host macrosystem and an- interdependence between them must exist. The concept of the influence of the macroenvironment in tumor development is consistent with the Darwinian theory of evolution that postulates selection as a fundamental property of biological systems. As environmental forces may explain breast cancer incidence patterns in the world population, host environmental factors may drive breast cancer phenotypes. Recent data indicate that some known risk factors for breast cancer are indeed systemic conditions such as obesity or diabetes. The epidemic prevalence of obesity and its associated comorbidities have been investigated as possible reasons for the increased incidence of cancer in Western societies. Although altered lipid profiles, including hypercholesterolemia, are extremely prevalent in these areas the role of systemic lipid metabolism in breast cancer is poorly understood.

Lipids are fundamental to cells, where they act either as constituents of the cell or as cell signaling molecules. Cholesterol, in particular, is an essential component of cell membrane bilayer, where it concentrates on the so-called lipid rafts-membrane microdomains which are involved in cell polarity, migration, proliferation, survival and angiogenesis. Cholesterol is also an obligatory precursor of steroid hormones, such as estrogen and progesterone, being the vast majority of breast tumors hormone responsive.

To date, most studies have seek to find a causal relation between cancer incidence and cholesterol plasma levels, however, fewer studies adressed a possible link in tumor aggressiveness or on its progression. Thus, for now, the importance of plasma cholesterol in breast cancer progression is largely unknown.

This Thesis aimed to study the role of systemic cholesterol in breast cancer progression and the underlying molecular mechanisms.

To do that, an observational prospective study was designed to follow a cohort of women with breast cancer (stages I-III), without previous treatment. Results show that plasma LDL-C at diagnosis positively correlates with tumor size and stage. At 2 years of follow up, higher levels of LDL-C were associated with reduced disease-free survival. These data indicate LDL-C as a biomarker of tumor aggressiveness (prognostic factor) and a possible therapeutic target (predictive factor).

To demonstrate a causal implication of cholesterol-enriched environment in tumor progression, well established *in vitro* and *in vivo* models were used. Results revealed that LDL-C signaling induces a tumor phenotype, also observed in breast cancer patients characterized by increased cell proliferation and invasion. Cells exposed to higher LDL-C concentrations have distinct genetic expression, supporting an aggressive phenotype, as confirmed by gene expression analysis. It was also experimentally shown that tumor ABCA1 (main cellular membrane cholesterol exporter) expression in cholesterol-enriched environment is a marker of LDL-C induced phenotype.

Together, the results of this thesis illustrate the influence of systemic metabolism in breast cancer progression and suggest that lipid profile must be assessed in all breast cancer patients. Being hypercholesterolemia so prevalent in Western societies with a global incidence pattern superimposing that of breast cancer, the modulation of cholesterol plasma levels are expected to have a major impact in (secondary) prevention. This seems particularly relevant for patients with hormone unresponsive tumors, for which no chemopreventive strategy exists.

Key words: breast cancer, cholesterol, LDL-C

Abbreviations

°C: degrees Celsius

µl: microliter

µM: micromolar

ABCA1: ATP-binding cassette protein A1

ABCG1: ATP-binding cassette protein G1

ACAT-1: acetyl-CoA acetyltransferase 1

AJCC: American Joint Committee on Cancer

Akt: Akt protein kinase

AMPK: mitogen activated protein kinase

ANOVA: analysis of variance

ATP: adenosine 5′-triphosphate

BMDC: bone marrow derived cells

BMI: body mass index

CDC: Centers for Disease Control and Prevention

cDNA: complementary deoxyribonucleic acid

CI: confidence interval

c-Myc v-myc avian myelocytomatosis viral oncogene homolog protein

c-myc-: v-myc avian myelocytomatosis viral oncogene homolog

CO_2: carbon dioxide

COX-2: cyclooxygenase-2

CXCR4: C-X-C chemokine receptor type 4

DAPI: 4',6-diamidino-2-phenylindole

DCIS: ductal carcinoma in situ

DFS: disease-free survival

DHCR7: 7-dehydrocholesterol reductase

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epithelial mesenchymal transition

ER: estrogen receptor

ERBB: epidermal growth factor B

ERBB2 (=HER2; =Her2-neu receptor): human epidermal growth factor receptor-2 or v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2

ERK: extracellular-signal-regulated kinases

FAK: focal adhesion kinase

Fas: CD95 or apoptosis antigen 1 (APO-1)

FBS: fetal bovine serum

FBSLF: fetal bovine serum lipoprotein free

FDG-PET: F-18 fluoro-2-deoxyglucose PET

FU: follow-up

g: gram

GFP: green fluorescent protein

GLY: glyburide

Gy: gray

H&E: hematoxylin and eosin

h: hours

HD: hypercholesterolemic diet

HDL (=HDL-C): high-density lipoprotein

HIF-1: hypoxia-inducible factor 1

HMGR: hidroxi-3-methyl-glutaril-CoA reductase

HR: hazard ratio;

IDL: intermediate-density lipoprotein

IGF-1R: insulin-like growth factor 1 receptor

IHC: immunohistochemistry

IPOLFG: Instituto Português de Oncologia de Lisboa, Francisco Gentil

JNK: jun amino-terminal kinase

Ki-67: antigen identified by monoclonal antibody Ki-67

LDL (=LDL-C): low-density lipoprotein

LDLR: low-density lipoprotein receptor

LRP1: low density lipoprotein receptor-related protein 1

LVI: lymphovascular invasion

LXR: liver X receptors α (LXRα) and β (LXRβ)

MAPK: mitogen activated protein kinase

mg: milligram

microRNA (=miRNA): micro ribonucleic acid

mL: milliliter

mm: millimeter

mM: millimolar

MMP: matrix metalloproteinase

mRNA: messenger ribonucleic acid

mTOR: mammalian target of rapamycin

NCCN: National Comprehensive Cancer Network

ND: normal diet

NFκB: nuclear factor kappa-light-chain-enhancer of activated B

NOS: not otherwise specified

NSAID's: non-steroid anti-inflammatory drugs

OLR1: ox-LDL receptor

OR: odds ratio

OS: overall survival

P: p-value

p53: tumor supressor protein p53

PAGE: polyacrylamide gel electrophoresis

PAI-1: plasminogen activator inhibitor type 1

PCR: polymerase chain reaction

PDGFR: platelet-derived growth factor receptor

PI3K: phosphoinositide 3-kinase

PLC: phospholipase C

PR: progesterone receptor

PTEN: phosphatase and tensin homolog

PUFA: polyunsaturated fatty acids

RB: retinoblastoma-associated gene

Rb: retinoblastoma-associated protein

RE: endoplasmasmic reticulum

RIPA: radioimmunoprecipitation assay

RNA: ribonucleic acid

ROS/RNS: reactive oxygen species/reactive nitrogen species

RR: relative risk;

RTC: randomized control trials

SCAP: sterol regulatory element-binding protein cleavage activating protein

SDF-1: stromal cell-derived factor 1

SDS: sodium dodecyl sulfate

SEM: standard error of the mean

SFK: Src family of kinases

Shh Sonic hedgehog protein

siRNA: small interfering ribonucleic acid

SOS: son of sevenless

SR-A1/2: scavenger receptors class A

SR-BI: scavenger receptors class B

SREBP: sterol regulatory element-binding protein

TC: total cholesterol

TGFα: transforming growth factor alfa

TGF-β: transforming growth factor beta

Tie-2: angiopoietin receptor 2

TNF: tumor necrosis factor

TP53: tumor suppressor protein p53 gene

uPA: urokinase-type plasminogen activator

uPAR: urokinase-type plasminogen activator receptor

VEGF-A: vascular endothelial growth factor-A

VLDL: very low-density lipoprotein

WHO: World Health Organization

WNT: wingless-type MMTV integration site family

ZEB1: E-box binding homeobox 1

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General Introduction

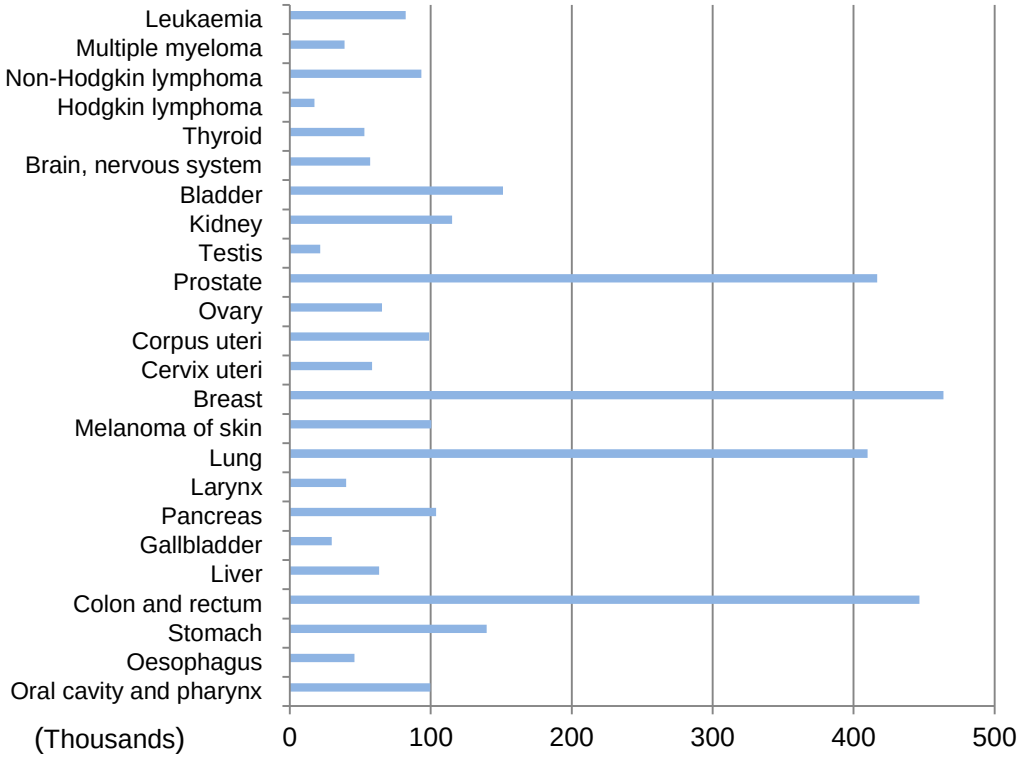
The general introduction of this Thesis is organized in two parts. The first one reviews the actual knowledge of breast cancer ethiopathogenesis and highligths some gaps in breast cancer research. The second part, briefly describes cholesterol functions and metabolism exploring published data on the relationship between cholesterol and breast cancer.

I. Breast cancer–The problem

Incidence and mortality

Cancer and cardiovascular diseases are the leading causes of death in the West[1,2] and are dramatically rising in Asian countries[3,4].

During 2012, there were over 3,4 million new cases of cancer, in Europe (excluding skin non-melanoma cancers) (Graphic 1). The most common cancer site was breast (464,000 cases, 13,5% of all cancer cases) and among women, breast cancer was the leading cause of death (131,000, 16,8% of all cancer)[5]



Graphic 1: Estimated number of new cases from cancer by site in Europe in 2012.
(Data from J. Ferlay et al, ,2013[6].)

In Portugal, breast cancer is the most frequent cancer in women, and despite being the cancer specific site (excluding skin cancers) responsible for the largest volume of oncologic treatments, including oncologic surgery, it still is the first cause of cancer-related death[7].

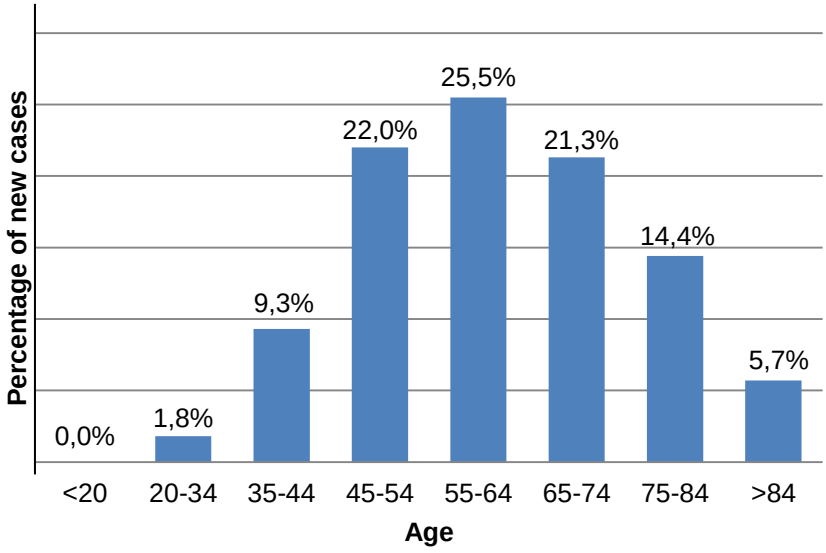
Faster diagnosis and progresses in systemic treatment have improved survival rates in the last three decades[8,9,10], but advanced cases (stage IV) have 5-year survival rate of 25%[11]. Identifying cases that will progress and that should be treated aggressively remains a major problem.

Etiopathogenesis

Breast cancer has been attributed to a combination of genetic susceptibility and other patient factors including age, reproductive, hormonal and lifestyle features.

Age

Breast cancer is extremely rare among women younger than 20 years old and is uncommon among women younger than 30 years old. Incidence increases sharply with age, being two thirds of invasive breast cancers found in women older than 55 years[11](Graphic 2).



Graphic 2: Percentage of new cases of breast cancer, by age.

Percentage of new cases in females of all races, 2007-2011. Data from Surveillance, Epidemiology and End Results Program (www.seer.cancer.gov)[11].

Genetic factors

By 1980, a significant body of evidence supported the presence of inherited factors responsible for familial clustering of breast cancer[12], and scientific efforts shifted to determine its inheritance pattern. Thirty years later, high-penetrance cancer susceptibility genes appear directly responsible for only 5-10% of all breast cancers. BRCA1 and BRCA2 gene mutations are the most common and well-studied genes associated with inherited breast cancer and account for almost all these cases[13]. Mutations in p53 (Li-Fraumeni syndrome[14]), PTEN (Cowden syndrome[15]), CHEK2[16], STKI/LKB1 (Peutz-Jeghers syndrome[17]), ATM[18], MSH2/MLH1 (Muir-Torre syndrome[19]), PALB2[20], BRIP1[21], were also described in breast cancer families but are less common.

The search for low penetrance alleles, either by candidate gene approach or genome wide association studies, did not produce major clinical impact. Apart from the identification of some loci strongly associated with breast cancer risk[22,23,24], the understanding how this knowledge can be applied clinically, is still missing. On the other hand, the identification of small interfering ribonucleic acids (siRNA) uncovers a new level of regulation of gene expression. Ribonucleic acids (RNAs) are the direct products of genes, and these small RNAs, also called micro RNAs (miRNAs) can bind to other specific messenger ribonucleic acid (mRNA) molecules and either increase or decrease their activity, resulting in gene silencing via translational repression or target degradation, thus, preventing protein expression[25]. It is now believed that as much as 92% of gene expression is regulated by siRNAs[26]. In breast cancer miRNAs show differential expression across molecular subtypes, and show both oncogenic and tumor-suppressive roles dependent on the context[27].

Non-genetic factors

Reproductive factors, hormonal factors and nutritional/lifestyle are considered non-genetic breast cancer risk factors. Whereas the causal effect of some of them is accepted, their individual strength and how they correlated with each other is more difficult to define.

Reproductive factors

Early age menarche[28], late menopause[29] and nuliparity[28], have all been consistently associated with increased risk of breast cancer[28]. Most studies point the early onset of ovulatory menstrual cycles and the greater lifetime exposure to endogenous hormones to be the reason of such association[30]. First pregnancy leads to proliferation of breast cells, which results in differentiation into mature cells prepared to lactation. After differentiation, epithelial cells have a longer cell cycle and spend more time in the G1 phase allowing for DNA repair[31]. In the same trend, lactation may be a protective factor because breast-

feeding results in further terminal differentiation of the breast epithelium and delays the return of ovulatory menstrual cycles after pregnancy.

Hormonal factors

Several lines of evidence have long suggested that sex hormones play a central role in the etiology of breast cancer. In animals, estrogens, progesterone, and prolactin all promote mammary tumors. Data from randomized controlled trials (RTC) have confirmed the epidemiologic relations of hormone therapy to increased risk of breast cancer and estrogen plus progestin therapy is now classified as a human carcinogen[32,33,34]. Conversely, hormonal manipulations, such as anti-estrogens and aromatase inhibitors are useful in the treatment of breast cancer and in reducing breast cancer incidence in high risk women[35,36,37]..

Nutrition/ lifestyle factors

Around the world, nutritional factors have been prominent among the hypothesized environmental determinants of breast cancer incidence and in the large increase in rates of migrant populations from low-incidence to high-incidence countries[38,39]. The dominant hypothesis has been that high-fat intake increases the risk of breast cancer. In agreement, overweight and obesity were clearly associated with increased incidence of breast cancer in postmenopausal women and a higher mortality rates[40,41,42,43]. Studies specifically addressing high-fat diet in breast cancer risk, showed increased occurrence of mammary tumors in rodents[44,45] although some of these studies loses the strong association when adjusted to energy intake[46]. Human ecological studies evidences that fat consumption *per capita* is highly correlated with breast cancer mortality rates[47]; and intervention studies show that the implementation of weight loss and low fat intake programs reduce breast cancer risk in 9% between interventional and control groups[48].

Other dietary elements possibly associated to breast cancer, include a positive association with alcohol consume[49] and a negative association with vitamin D[50,51,52] intake.

Physical exercise is suggested as protective[53], through body mass index (BMI) reduction, menarche delay, sex hormones and insulin-like growth factors lowering and by improving immune function[54, 55].

Other factors

Other variables searched for a relation with breast cancer risk include; proliferative breast diseases, ionizing radiations, active and passive smoking, silicone breast implants, diabetes mellitus, thyroid cancer, non-steroid anti-inflammatory drugs (NSAID's), statins and antidepressants use. As for comproved risk factors, the relative weight of each factor

is hard to predict in the individual case (Table 1) and for some of them the literature is very scarce.

Proliferative breast diseases without atypia slightly increases the risk of breast cancer while atypical hyperplasia represents a moderate increased risk of breast cancer applying for both glands even when it is only unilateral[56].

Ionizing radiation to the chest, in cumulative doses at young age, substantially increases breast cancer risk. Evidence on this topic comes from atomic bomb survivors[57], therapeutic[58] and occupational[59] radiation use studies. The regular diagnostic radiation use, such as the mammographic screening, shows no significant increased risk of breast cancer[60].

Recent studies have shown the association of type 2 diabetes with the incidence of breast cancer[61,62,63] and cancer-specific mortality. This association was most pronounced in postmenopausal women and ER-positive disease[63]. This effect is attributed to insulin, which acts as a breast cancer cell growth factor[64,65]. But because many other conditions lead to hyperinsulinemia, further studies on the relationship between breast cancer and insulin resistance are warranted. Nevertheless, clinical trials with oral anti-diabetics drugs are in course[66].

Table 1: Risk factors for breast cancer and approximate strength of association

Reproductive Factors	**Hormonal Factors**	**Nutritional/Lifestyle Factors**	**Others Factors**
Early age at menarche +	Oral contraceptives use (current vs none) +	Obesity (BMI>30vs>25) Premenopausal – Postmenopausal +	Family history (mother and sister) +++
Age at first birth (>35vs<20) ++	Estrogen replacement (10 years vs none) +	Adult weight gain (postmenopausal) ++	Family history (1st–degree relative) ++
No. of births (0 vs 1 child) +	Hormone replacement (>5 years vs none) ++	Alcohol (one or more drink vs none) +	Jewish heritage (yes vs no) +
Age at menopause (5-years increment) +	↑ blood estr./andr (post menopause) +++	Physical activity (>3hours/week) -	Ionizing radiation (yes vs no) +
Breast-feeding (>1year vs none) -	High blood prolactin ++	Monounsaturated fat (vs saturated fat) -	Benign breast disease ++

- no risk; +low risk; ++moderate risk; +++high risk

Although the relative strength of known or suspected breast cancer risk factors are modest in magnitude (RR are usually in the range of 1.3 to 1,8) the impact of risk factors control could be very large. When considering (primary) prevention, it is important to remember that even small changes at individual level can produce substantial changes in population rates of disease[67]. A recent study estimates that up to 27% of breast cancer-related deaths would be avoided with control of key behavioral and environmental risk factors such as alcohol use, overweight and obesity and physical inactivity[68].

Nevertheless, only about 30% of breast cancers are estimated to be explained by known risk factors[69], leading to the hypothesis that other environmental factors could play a major role. On the other hand, mechanisms linking known and suspected risk factors to the initiation and development of breast cancer are poorly understood. Therefore, there may still be a large amount of unknown risk factors to breast cancer etiopathogenesis.

Phenotypes

Invasive breast cancers are a heterogeneous group of lesions differing with regard to their clinical presentation, imagiological appearance, pathological features, gene expression profiling and biological behavior.

The phenotype is usually based on the histological characteristics of the tumor, enclosing a range of genotypes and biological behaviors. In clinical practice, the phenotype is used to define therapy and prognosis.

Based on World Health Organization (WHO)[70], the most widely used histological classification system of breast cancers, the most common histological type is invasive (infiltrating) ductal carcinoma (53-70%), recently denominated invasive carcinoma, not otherwise specified (NOS). The other types comprises a group of invasive breast cancers with specific or special histological features: invasive lobular carcinoma (5-16%), medullar (3-9%), tubular (1-3%), mucinous (1-2%), and other rare types[71,72,73]. Non-epithelial breast cancers such as sarcomas or lymphomas are even rarer.

The routine pathologic examination of invasive breast cancers defines the histological type and the grade. Histological grade is based on the degree of differentiation of the tumor tissue. For breast cancer the most reproduced system is the Nottingham (Elston-Ellis) grade system[74]. It refers to the semi-quantitative evaluation of three morphological characteristics: degree of tubule or gland formation, nuclear pleomorphism and mitotic count. The final score shows a very strong correlation with prognosis; patients with grade I tumors have a significantly better survival than those with grade II and III tumors[75].

Additionally, three immunohistochemical markers (estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (ERBB2 or Her-2/neu receptor or HER2)) are generally used to identify biological properties. And in the last years, proliferative markers such as Ki67 are also used to determine tumor index proliferation rate, since it was demonstrated to have correlation with prognosis[76].

The tumor subgroups identified by immunohistochemistry closely resemble the molecular subtypes, defined by Sorlie et al, based on gene expression profiling studies: Luminal A, Luminal B, HER2 type and basal-like[77].

Luminal A and luminal B cancers generally have a good prognosis and show high expression of hormone receptors and associated genes. Together, these two subtypes account for approximately 70% of all breast cancers. The luminal B cancers tend to be higher grade than the luminal A and some of them may overexpress HER2. Both luminal A and luminal B cancers usually respond to hormone therapy, with luminal A cancers showing the improved response. Response of the luminal cancers to chemotherapy is variable, with the luminal B cancers generally showing better response.

The HER2 type cancers show high expression of HER2 and low expression of ER and associated genes. They account for approximately 15% of all breast cancers and are generally ER or PR negative. HER2 cancers are more likely to be high grade and have positive lymph nodes. These cancers show the best response to trastuzumab and to anthracycline-based chemotherapy but, overall have a poor survival prognosis.

The basal-like breast cancers show high expression of basal epithelial genes and basal cytokeratins, low expression of ER and ER associated genes as well as low expression of HER2. They constitute approximately 15% of all breast cancers and are often referred to as triple negative cancers, because they are invariably ER, PR, and HER2 negative. The basal-like tumor phenotype is especially common in African-American women and is also the most common phenotype of BRCA1-associated breast cancers. Basal-like cancers have a poor prognosis and are not amenable to treatment with either hormonal or biological therapy.

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Prognostic and predictive markers

Despite usefulness of pathological characteristics, in which clinical practice and therapeutic decision are based, they do not linearly correlate with tumor biology and

behavior. In order to define prognosis and predict therapeutic response, clinicians classify a newly diagnosed breast cancer through stage and a panel of associated factors.

The American Joint Committee on Cancer (AJCC) system is the most used to stage tumors. It is both a clinical and pathological staging and is based on the TNM system, in which T refers to tumor, N to regional lymph nodes, and M to distant metastasis[78]

At present, the prognostic and predictive factors of primary breast cancers used in clinical practice are[79]:

1. Axillary lymph node status, including micrometastasis (Nmic)

2. Tumor size

3. Histological subtype

4. Histological grade

5. Proliferation indices, including mitotic index

6. Estrogen and progesterone receptor status (mainly as predictive markers of response to hormonal therapies)

7. HER2 amplification or overexpression (mainly as a predictive marker of response to trastuzumab and possibly as predictive of benefit from anthracyclines)

8. Multiparameter-based markers (mainly as prognostic indicator of recurrence risk).

The identification of a single biomarker to predict prognosis and response to therapy would be a major step forward in oncology. However, one of the hallmarks of cancer is the redundancy of cellular pathways, leading this single biomarker difficult to obtain.

In the last decades technology for individualizing therapy on the basis of gene arrays for tumor characteristics has bloomed and was heralded to dictate the future individualized therapy. For instance, gene arrays, also termed gene expression arrays or DNA microarrays, are a method to simultaneously determine the expression levels of up to 25,000 human genes in a tumor or normal tissue simultaneously. Among several gene expression based prognosticators[80,81,82] only few was validated, but they do not replace traditional prognostic factors. Even the most validated assays have been studied only in relatively small datasets or as subsets of larger clinical trials and many of them have not been validated at all on independent test sets. Moreover, the field of breast cancer therapy is rapidly changing and the natural history of breast cancer can be very long. In

these circumstances the evidence on prognosis and efficacy of a given approach can be obsolete during development of validation studies.

On opposition relation of tumor with other host pathological or physiological pathways has barely been explored. For example, the effectiveness of certain drugs, such as tamoxifen and the chemotherapy irinotecan, is mediated by metabolism of cytochrome P450 enzymes, mainly in the liver. Based on genetic polymorphisms the activity of such enzyme complex have considerable inter-individual variability[83]. This aspect may contribute to the observed variability in the response to hormone therapy. This underlines the dependency of the host and how systemic characteristics need to be considered for tumor biology interpretation and treatment strategy.

The future of prognostication and prediction may rely on the integration of classic biomarkers, such as ER status and stage, with genomic biomarkers and individual characteristics.

Tumor biology

Normal cells became neoplastic and lead to the onset of cancer by progressively acquiring hallmark capabilities such as: sustained proliferative signaling, cell death resistance, evasion to growth suppressors, replicative immortality and angiogenic and invasion (metastastatic) potential. Reprogramming energy metabolism and evasion from immune destruction are now being considered as emerging hallmarks[84].

Breast cancer proliferation genetics and signaling pathways

Proliferation signals are conveyed in large part by growth factors that bind cell-surface receptors, typically containing intracellular tyrosine kinase domains. These emit signals via branched intracellular signaling pathways that regulate cell cycle, progression and proliferation. Often, these signals influence other cell-biological properties, such as cell survival and energy metabolism.

Cancer cells may produce growth factor ligands themselves, resulting in autocrine proliferative stimulation or, alternatively send signals to stimulate normal cells within the supporting tumor-associated stroma, which supply cancer cells with various growth factors[85,86]. Receptor signaling can also be deregulated by elevating the levels of receptor proteins at the cancer cell surface, rendering such cells hyperresponsive. Growth factor independence may also derive from the structural alterations in the receptor molecules or constitutive activation of elements of signaling pathways operating downstream of these

receptors. Given that a number of distinct downstream signaling pathways radiate from a ligand-stimulated receptor, the activation of one or another of these downstream pathways may only recapitulate a subset of the regulatory instructions transmitted by an activated receptor. In breast cancer, some of these mechanisms have been demonstrated. Estrogen and progesterone induce proliferation and differentiation of normal breast epithelium. Their effects are mediated trough ER and PR, respectively. ER is highly elevated in nearly all precursors[87,88] and drugs targeting this receptor (e.g. tamoxifen) reduce breast cancer by 50%[36,89]. Nuclear/genomic ER can activate growth factor pathways by increasing the expression of ligands (transforming growth factor α-TGFα, amphiregulin), receptors (insulin-like growth factor 1 receptor -IGF-1R), or other signaling intermediate molecules (insulin receptor substrate-1) which are estrogen regulated and important for growth factor activity[90]. In addition to the genomic activity, ER has a non-genomic action by membrane initiated steroid signaling[91]. Membrane ER may exist as a cytoplasmic entity tethered to the inner face of the plasma membrane bilayer through binding to proteins of lipid rafts, scaffold or adaptor proteins[92,93], or possibly associating with other membrane receptors, such as IGF-1R[94,95] epidermal growth factor receptor (EGFR)[96], or ERBB2[92,97].

ERBB2 (also known as *neu* oncogene or HER2) amplification, with resultant ERBB2 protein overexpression, has been shown to play a role in sustaining multiple cancer pathways, including self-sufficiency in growth signals, sustained angiogenesis, increased cell division, and enhanced invasion[98,99,100]. ERBB2 receptor (amplifications or overexpression)[101] are present in 15-20% of breast cancers and inhibition of ERBB2 membrane signaling in these cancer cells through administration of humanized anti-ERBB2 antibodies (trastuzumab) or administration of small molecule inhibitors of ERBB2 tyrosine kinase activity (lapatinib) is associated with improved patient outcomes for women with both primary and metastatic disease[102,103,104].

Constitutive activation of signaling circuits usually triggered by activated growth factor receptors is also a cancer cell mechanism to proliferate. Mutations in the phosphoinositide 3-kinase (PI3K) pathway are frequent in breast cancer, causing resistance to ERBB2-targeting agents and, possibly, to hormonal agents as well. Multiple PI3K inhibitors are currently under development, including pure PI3K inhibitors, compounds that block both PI3K and mammalian target of rapamycin receptor (mTOR) (dual inhibitors), pure catalytic mTOR inhibitors, and inhibitors that block Akt[105].

Disruptions of negative-feedback mechanisms that attenuate proliferative signaling are another way of tumor cell perpetuates proliferation. Defects in ras GTPase, phosphatase and tensin homolog (PTEN) phosphatase and mTOR kinase are examples[105] (Figure 1).

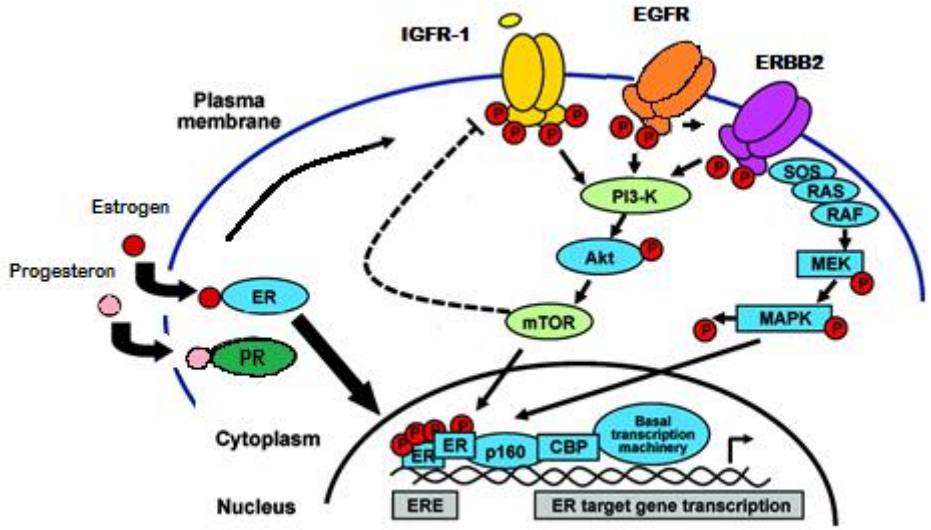


Figure 1: Major pathways regulating proliferation of breast cancer cells.

Abbreviations: CBP:CREB binding protein; ER:estrogen receptor; ERE:estrogen-responsive element; HER2: human epidermal growth factor receptor 2; MAPK:mitogen-activated protein kinase; MEK:mitogen-activated protein kinase/extracellular signal–related kinase kinase; mTOR:mammalian target of rapamycin; PI3K:phosphoinositide-3kinase; SOS:son of sevenless. (Adaptated from Di Cosimo S et al, Management of breast cancer with targeted agents: Importance of heterogenicity,2010[106])

In addition, evading growth suppressors will also promote proliferation. The two prototypical tumor suppressors encode retinoblastoma-associated protein (Rb) and tumor suppressor protein p53 (p53). TP53 (p53 gene) mutations are common in precursors lesions and breast cancer and are supposed to be the cause of 1% of hereditary cases[13,107].

Growth factor–dependent and hormone-dependent signaling are not the only important signal transduction pathways that derive from the cell surface. Two important classes of adhesion-dependent signals also play roles in cell growth, differentiation, and survival, as well as in motility and invasion of breast cancer cells: integrin and cadherin adhesion molecules[108,109].

Integrins carry signals from the extracellular matrix into the cell. Integrins signal cells through many pathways, but one that has received considerable current interest is the focal adhesion kinase (Fak)-Src pathway. Activation of these two tyrosine-kinase–encoding oncoproteins activates the PI3K and Akt candidate oncoproteins, leading to multiple aspects of malignant behavior[108,109]. Of considerable interest, the tumor-suppressive phosphatase PTEN acts on Fak, PI3K, and Akt as its substrates to suppress

survival and induce apoptosis. However, mutations of PTEN do not appear to be particularly common in sporadic breast cancer[110,111].

One of the functions of E-cadherin is to restrict cell motility, but it also diminishes the cytoplasmic pool of the important proliferation-modulating βcatenin transcription factor. The tumor-suppressive E-cadherin gene is commonly silenced by DNA methylation or mutation in breast cancer, releasing βcatenin to potentially up-regulate expression of the c-*myc* protooncogene (v-myc avian myelocytomatosis viral oncogene homolog)[112,113]. The βcatenin pathway may also be up-regulated by growth factor pathways, such as epidermal growth factor (EGF), hyperglycemic-glycogenolytic factor, and WNT (wingless) signaling[114].

Cell death mechanisms

By 2000, it was widely accepted that cancer cells require unlimited replicative potential in order to generate macroscopic tumors. However, a balance between replication and death is required and involved in cancer progression. Cell death can occur by several mechanism including apoptosis or oncosis.

The concept that programmed cell death by apoptosis serves as a natural barrier to cancer development has been established by compelling functional studies [115,116,117]. The apoptotic machinery is composed of both upstream regulators and downstream effector components[115]. The regulators, in turn, are divided into two major circuits, one receiving and processing extracellular death-inducing signals (the extrinsic apoptotic program, involving for example the Fas ligand/Fas receptor), and the other sensing and integrating a variety of signals of intracellular origin (the intrinsic program). Each one culminates in the activation of a normally latent proteases (caspases 8 and 9), that proceed to initiate a cascade of proteolysis involving effector caspases responsible for the execution phase of apoptosis, in which the cell is progressively disassembled and then consumed, both by its neighbors and by professional phagocytic cells. The ‘‘apoptotic trigger’’ that conveys signals between the regulators and effectors is controlled by counterbalancing pro- and anti-apoptotic members of the Bcl-2 family of regulatory proteins[115]. Although the cellular conditions that trigger apoptosis remain to be fully enumerated, several abnormality sensors that play key roles in tumor development have been identified[115,116]. Most notable is DNA damage sensor that functions via the p53 tumor suppressor protein[118]. As referred previously, mutations in p53 leads tumor cells to evade growth suppression.

Trigger apoptosis signaling circuit is the mechanism of action of some anticancer therapy but is also a response to various physiological stresses that cancer cells experience

during the course of tumorigenesis and is substantially elevated in proliferative lesion such as higher grade DCIS[119,120]. This exposes that much more remains to elucidate about this process.

In contrast to apoptosis, cells dying by oncosis (commonly denominated necrosis, which refers just to the pathological aspect of cell death) become bloated and explode, releasing their contents into the local tissue microenvironment. Although oncosis has historically been viewed much like organismic death, as a form of system-wide exhaustion and breakdown, the conceptual landscape is changing: cell death by necrosis is clearly under genetic control in some circumstances, rather than being a random and undirected process[121,122]. Perhaps more important, necrotic cell death releases pro-inflammatory signals into the surrounding tissue microenvironment, in contrast to apoptosis and autophagy, which do not. Consequently, necrotic cells can recruit inflammatory cells of the immune system. Multiple lines of evidence indicate that immune inflammatory cells can promote tumor progression, given that such cells are capable of fostering angiogenesis, cancer cell proliferation, and invasiveness[123,121].

Autophagy is a process by which cells clear damaged or superfluous proteins and organelles. The recycling of these intracellular constituents also serves as an alternative energy source during periods of metabolic stress to maintain homeostasis and viability. Although not a cell death process, recent evidence suggests that autophagy provides a protective function to limit tumor necrosis and inflammation, and to mitigate genome damage in response to metabolic stress and defects in apoptosis[124]. The mechanism behind this has not been totally determined but is expected to be an anticancer target in near future[125].

Tumor neo-vascularization

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs. During embryogenesis, the development of the vasculature involves the generation of new endothelial cells and their assembly into tubes (vasculogenesis) in addition to the sprouting (angiogenesis) of new vessels from existing ones. Following this morphogenesis, the normal vasculature becomes largely quiescent. In the adult, in physiological processes such as wound healing and female reproductive cycling, angiogenesis is turned on, but only transiently. In contrast, during tumor progression, an ‘‘angiogenic switch’’ is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help to sustain expanding

neoplastic growth[126]. A compelling body of evidence indicates that the angiogenic switch is governed by factors that either induce or oppose angiogenesis[127,128]. The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1, respectively[129]. Histological studies of premalignant, noninvasive lesions, including dysplasias and *in situ* carcinomas arising in a variety of organs, have revealed the early triggering of the angiogenic switch[126]. Although angiogenesis is well documented in breast cancer progression anti-angiogenic drugs failed to show the expected clinical success[130].

Cancer metabolism

The chronic and often uncontrolled cell proliferation that represents the essence of neoplastic disease involves not only deregulated control of cell proliferation but also corresponding adjustments of energy metabolism in order to fuel cell growth and division. The existence of this metabolic switch in cancer cells was recognized since the pioneering work of Otto Warburg in the first half of the twentieth century[131]. Despite this feature has received little or no attention for decades, it has been the object of major research effort in recent years[132,133].

Cancer cells exhibit increased nutrient uptake

Cancer cells increase their glucose uptake, but instead of oxidizing most of this glucose to efficiently generate adenosine 5′-triphosphate (ATP) by oxidative phosphorylation, they ferment the excess glucose to lactate. This phenomenon is observed even in the presence of oxygen, and is referred as the Warburg effect or aerobic glycolysis[134,135](for review of molecular mechanism[136])(Figure 2). Previously, aerobic glycolysis was suggested to be a consequence of mitochondrial damage[131] or an adaptive response to tumor hypoxia[137]. However, mitochondria remain functional in most tumors, and aerobic glycolysis is observed in cancer cells even in normoxia[134,135].

Aerobic glycolysis, may allow individual cancer cells to increase uptake and incorporation of nutrients into the biomass (nucleotides, amino acids, and lipids) and facilitate the construction of new cells. According; 1) several signaling pathways implicated in cell proliferation also regulate metabolic pathways that incorporate nutrients into biomass; and 2) certain cancer-associated mutations enable cancer cells to metabolize nutrients in a manner conducive to proliferation rather than efficient ATP production. In support of this idea, aerobic glycolysis is a characteristic of many rapidly proliferating normal tissues and microorganisms[134]. Satisfying the metabolic needs of proliferation and redox control beyond ATP production may be advantages of aerobic glycolysis[138]. Moreover, studies have shown that lactate, a metabolite of aerobic glycolysis is also mediating the malignant

transformation and selection of surrounding cells leading to tumor progression and invasion[139,140].

Although glucose catabolism through aerobic glycolysis has in large part been recognized a hallmark of cancer, it alone cannot explain all the metabolic changes necessary to support the requirements of cell growth[141]. Many normal mammalian tissues use nutrients other than glucose, and consumption of alternative fuel sources is observed in some cancer cells. Glutamine is the most abundant amino acid in both serum and cell culture medium, and glutamine is an important source of nitrogen for cells[138,142]. The carbon skeleton of glutamine can be oxidized to generate ATP and replenish citrate cycle intermediates. Finally, in some contexts reductive glutamine metabolism can provide carbon for lipid synthesis[143,144]. Indeed, after glucose, glutamine is the nutrient most highly consumed by cancer cells in tissue culture[142,145]. Emerging evidence suggests that other nutrients, including fatty acids and other amino acids, can also play key roles in some contexts [145,146,147].

Increased nutrient uptake is exploited in the clinic as a way to image tumors. F-18 fluoro-2-deoxyglucose PET (FDG-PET) is used to visualize tumors activity. This technique serves as a measure of glucose uptake in patient tissues by coupling positron-emitting ^{18}F to an analog of glucose that is taken up and trapped in cells by phosphorylation but is not subject to further metabolism[148]. FDG-PET is most useful clinically as a staging tool and can also be used to monitor therapy response[149]. PET scanning to monitor uptake of other nutrients, such as glutamine and glutamate analogs has also been described in research settings[149].

Cancer cells use different metabolic programs

While cancer metabolism is often considered as a property that differs from normal cell metabolism, there is evidence that tumor cells exhibit a diversity of metabolic phenotypes[150,145,151,15

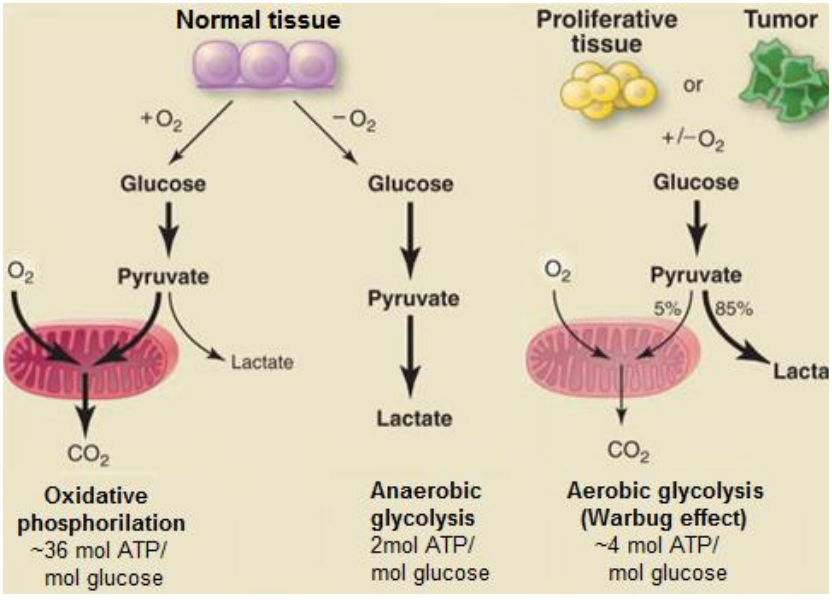


Figure 2: Glucose catabolism in normal and tumor cells

[2,153]. Heterogeneous expression of metabolic genes is observed across tissue types, and the metabolic network of an individual tumor more closely resembles that of the normal tissue from which the tumor arose than it does with other tumors that develop in different organ sites[150]. This expression pattern may reflect the propensity of cancer cells to adapt the pre-existing metabolic network to support their needs.

Indeed, the metabolic phenotype of tumors must be a function of both the genetic lesion driving tumorigenesis and the tissue from which the cancer arose[152]. Expression of oncogenes promotes increased consumption of glucose, glutamine, and proteins and can reprogram metabolism to support cell growth and proliferation[146,154,155,156]. Increasing evidence indicates that tumor suppressor genes function in part through effects on metabolism[157], and the combination of genetic mutations in specific tissues facilitates altered metabolic regulation to support abnormal tissue growth.

Tumor cell metabolism is influenced by external factors

Altered tumor metabolism is not simply the final outcome of some combination of cell genetic modifications. Instead, a non-genetic component in the form of the tumor microenvironment must additionally be considered as part of the equation that influences metabolic changes in cancer cells[158](Figure 3). Solid tumors are poorly vascularized, and therefore their surrounding environment can expose distinct regions of the tumor to spatial and temporal gradients of oxygenation, pH, and nutrient availability[159]. For example, fluctuating oxygen gradients across the microenvironment can drive sporadic hypoxia, the stabilization of hypoxia-inducible factor 1 (HIF1), and a corresponding induction of the HIF1-induced transcriptional program[160]. Regardless of whether HIF1-induced transcriptional effects are promoted through inappropriate genetic regulation or in response to hypoxic stress, one of its downstream consequences remains the conversion of a large percentage of glycolytic pyruvate to secreted lactate. The secreted lactate in turn triggers additional metabolic responses as a result of local acidification within the tumor microenvironment. It has also been suggested that this lactate-driven acidification can promote both tumor invasion and immune evasion[161,162], which are among the other denoted hallmarks of cancer. Moreover, lactate secretion may have a functional role within a larger system of metabolic cooperation and symbiosis between cells in the microenvironment. Described as a “2-compartment” model of tumor metabolism, the symbiosis is characterized as the potential for anabolic malignant cells to extract high-energy metabolites (lactate, glutamine, and fatty acids) from adjacent catabolic cells (within the tumor or neighboring stromal cells) through a network of nutrient sharing that can stimulate tumor proliferation and metastasis[163,164].

Studies reporting 2-compartment tumor metabolism have recently emerged in the context of breast cancer cells and their neighboring fibroblasts[165,166,167], as well as for ovarian cancer cells and their neighboring adipocytes[168]. However, the complex interplay between genetics, microenvironment, and tissue heterogeneity is poorly understood. In addition, whole body metabolic regulation can affect tumor tissue metabolism, and patients with cancer often have perturbations in whole body metabolism[169]. Altered organismal metabolism can affect cancer outcomes as evidenced by the relationships between cachexia[170,171], obesity[40] or diabetes[61] and poor patient survival.

Powerful homeostatic mechanisms exist at the organismal level to maintain a relatively constant supply of nutrients available to both normal and malignant tissues. This complex system cannot be understood from cell cultures studies or simple models and remains a challenge for the field (Figure 3).

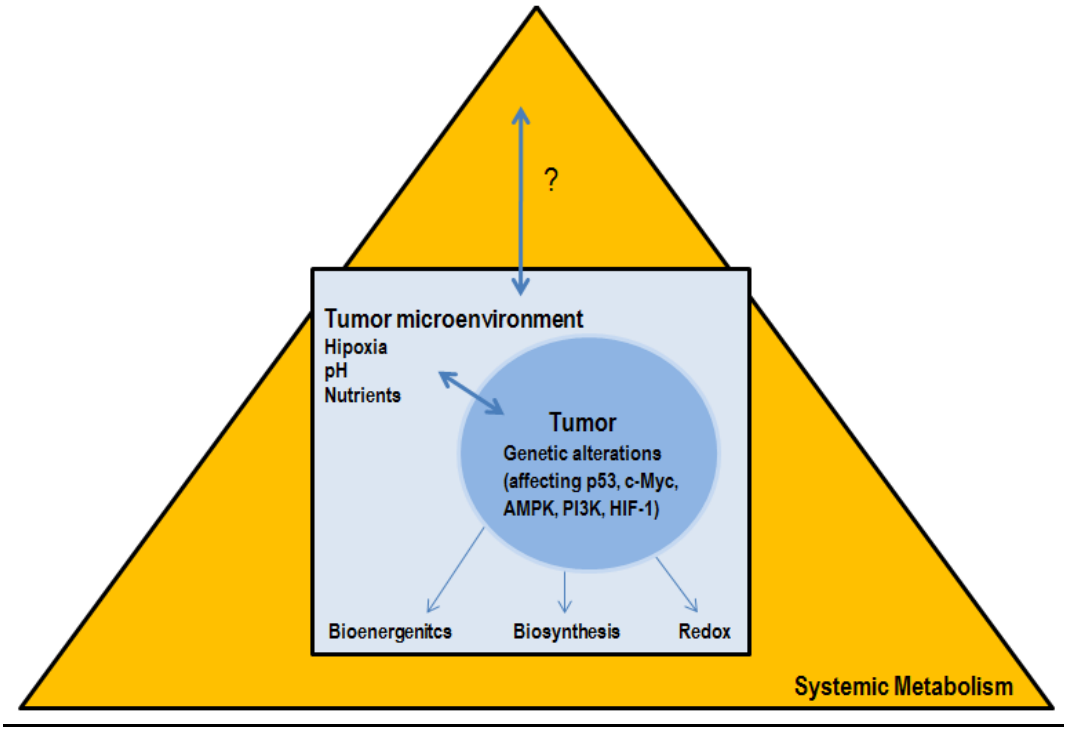


Figure 3: Determinants of the tumor metabolic phenotype.

The metabolic phenotype of tumor cells is controlled by intrinsic genetic mutations and external responses to the tumor environment. Oncogenic signaling pathways controlling growth and survival are often activated by the loss of tumor suppressors (such as p53) or the activation of oncoproteins (such as PI3K). The resulting altered signaling modifies cellular metabolism to match the requirements of the cell division. Abnormal microenvironment conditions such as hypoxia, low pH and or nutrient deprivation elicit responses from tumor cells, including autophagy, which further affect metabolic activity. These adaptations optimize tumor metabolism for proliferation by providing appropriate levels of energy in the form of ATP, biosynthetic capacity and the maintenance of balanced redox status. The influence of the systemic metabolism on this microsystem is not known. AMPK: AMP-activated protein kinase; HIF-1: hypoxia-inducible factor 1.

Evading the immune system

The long-standing theory of immune surveillance proposes that cells and tissues are constantly monitored by an ever-alert immune system, and that such immune surveillance is responsible for recognizing and eliminating the vast majority of incipient cancer cells and thus emerging tumors. According to this logic, solid tumors that do appear have somehow managed to avoid detection by the various components of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication. Although the supposed mutual dependencies of the immune system, other cancer hallmarks and systemic metabolism, this topic will not be further developed (for review[172]).

Tumor environment

The microenvironmental influence on tumor progression is well recognized and is known to contribute to cancers heterogeneity (for review[173]).

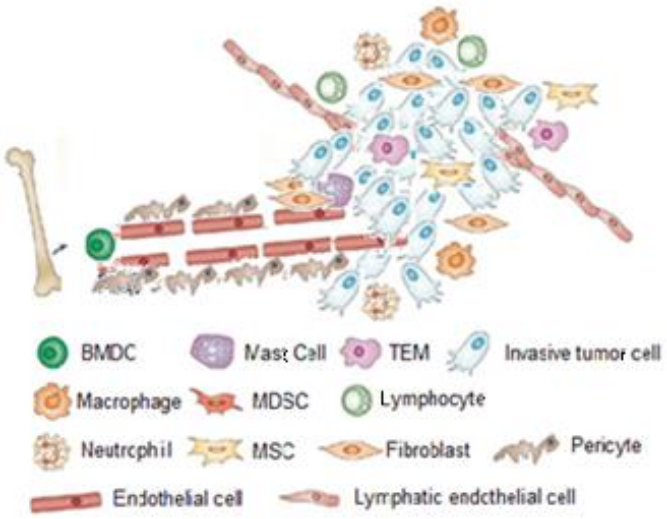


Figure 4: Primary tumor microenvironment

Cancer cells in primary tumors are surrounded by a complex microenvironment comprising numerous cells including endothelial cells of the blood and lymphatic circulation, pericytes, stromal fibroblasts and a variety of bone marrow-derived cells (BMDC), including macrophages, neutrophils, myeloid-derived suppressor cells, TIE2-expressing monocytes and mesenchymal stem cells. (Adapted from Joyce,JA et al, Microenvironmental regulation of metastasis, 2009)

Homeostasis in normal tissues requires a tightly controlled balance of cell proliferation and death, which is achieved and maintained through intercellular communication. An important regulator of normal cell behavior and tissue hemostasis is the surrounding extracellular matrix (ECM). The ECM has many functions, including acting as physical scaffold faciliting interactions between different cell types, providing survival and differentiation signals. Maintaining organ homeostasis can prevent neoplastic transformation in normal tissues by ensuring stable tissue structure, mediated by tight junction proteins and cell adhesion molecules such as integrins and E-cadherin[174,175]. During early tumor development, however the protective constrains of the microenvironment are overridden by conditions such as chronic inflammation, and local tissue microenvironment shifts to a growth-promoting state.

It is now well established that primary tumors comprise a multitude of stromal cell types in addition to cancer cells[176] (Figure 4). Although the majority of cell-cell interaction is still unknown, there is increasing evidence that the microenvironment induces important signals to tumor cells to develop mesenchymal properties (called epithelial to mesenchymal transition (EMT), see below), intravasation, survival in circulation and also tropism and proliferation in metastatic distant sites[173].

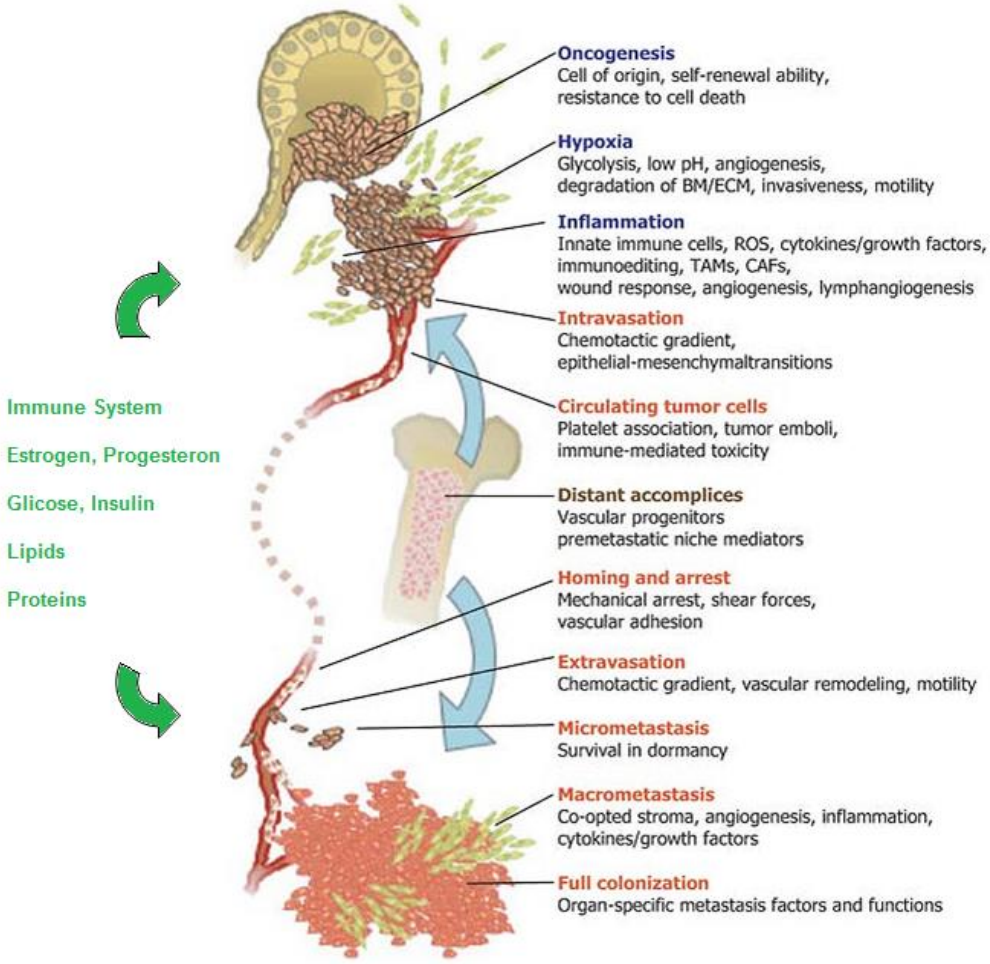


Figure 5: Selective pressures and steps from primary tumor growth to metastasis

Cancer is initiated by cell intrinsic oncogenic changes. Early events involve mutations of breast stem cells. These mutations can be inherited (e.g. mutations in BRAC1, BRAC2 or P53) or acquired as consequence of some exposure carcinogenic. Microenvironmental factors such as hypoxia, low pH, immune cells, and stromal cells have important roles in tumor progression. Bone marrow-derived cells (BMDC) not only home to the primary site to aid primary tumor growth, but also home to future distant sites of metastasis to form a pre-metastatic niche[179]. Together with new accomplices, angiogenesis, lymphangiogenesis, and invasion toward chemotactic gradients occur. Intravasation gives rise to circulating tumor cells and starts the metastatic cascade. Survival of these circulating cells necessitates resisting mechanical stress and cell-mediated toxicity. Sites of extravasation are biased by circulatory patterns, mechanical forces, and chemotactic gradients. Familiar challenges and elements faced in distant organs may allow growth. Alternatively, some selective pressures in metastatic sites will not be familiar and will limit full colonization, resulting in dormancy. However, these new selective pressures that are organ-specific can drive the acquisition of further genetic alterations. If successful, full colonization will occur. During all this process, systemic pressures may act in other to inhibit or facilitate tumor progression (green). In breast cancer, the target variables may include elements such as immune system, hormonal and metabolic resources availability.(Figure adapted from DeVita eta al, Cancer: Principles and Practice of Oncology,9th edition, Charper 9)

This “ecosystem” is contained within a macroenvironment system whose selective pressures are often forgotten (Figure 5). Illustrating the influence of such selective pressures are the partial responses to systemic therapy and the emerging resistance of some tumor subclones. Likewise, the association of a systemic condition and specific cancer phenotypes, such as obesity and ER negative breast cancers[177,178] or the fact that systemic estrogens stimulate ER positive breast cancers[32,33], support this notion of a macroenvironmental control of cancer progression.

Currently, the phenotype of the primary tumor and clinical staging are used to define prognosis and therapy. Great efforts have been made to integrate genotypic characteristics (genotype) in clinical guidelines; however, the systemic properties of the host, important in tumor progression and response to therapy have not been taken into account in this equation.

Tumor dissemination

How cancer cells invade surrounding tissues and disseminate to distant organs (metastasis) has been a matter of debate for decades.

Metastasis is the leading cause of breast cancer-related death[180]. One of the primary aims in cancer clinical management is to prevent or decrease the risk of metastasis. How this objective is approached is shaped by empiricism and perceptions about how metastasis proceeds because no single explanation of the full complexity of the metastatic process has been proven to be correct or complete.

Numerous prerequisites are needed for cells to become metastatic: 1) Invasion and motility–Tumor cells may undergo a epithelial to mesenchymal transition (EMT) to become motile, a fundamental property of metastatic cells, and use their migratory and invasive properties in order to burrow through surrounding extracellular stroma and to enter blood and lymphatics vessels; 2) Intravasation and survival in the circulation-Once tumor cells enter the circulation, or intravasate, they must be able to withstand the physical shear forces and the hostility of sentinel immune cells. Solid tumors are not adapted to survive as single cells without attachments and often interact with each other or blood elements to form intravascular tumor emboli.); 3) Arrest and extravasation-Once arrested in the capillary system of distant organs, tumor cells must extravasate, or exit the circulation, into foreign parenchyma. This may happen by physical means whereby intravascular growth causes eventual disruption of small capillaries, or escape may be regulated via

invasive properties acquired by tumor cells; 4) Growth in distant organs-Successful adaptation to the new microenvironment results in sustained growth.

Normal epithelial cells, such as breast ductal cells, are displayed in a tissue structure maintained by cell-cell interactions. These involve tight junctions, cadherin based adherens junctions that are connected to the actin cytoskeleton, gap junctions that allow direct chemical interactions between neighboring cells, and desmosomes connected to the intermediate filament cytoskeleton, and cell-ECM interactions mediated by integrins and other molecules. The cell-cell and cell-ECM contacts also define tissue polarity[181], which allows different functions for the apical and basal surfaces.

Epithelial to mesenchymal transition is a biologic process that allows these cells to undergo multiple biochemical changes that enable them to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM component[182]. This initiates the invasive and metastatic behavior of epithelial cancers, but EMT mechanisms are also recognized in physiological conditions such as during embryogenesis or tissue regeneration[183].

A number of distinct molecular processes are engaged in order to initiate an EMT and enable it to reach completion. These include activation of transcription factors (Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOXC2[184,185,186,187,188]), expression of specific cell-surface proteins (SMA, FSP1, vimentin, and desmin[189], β4integrin, α5β1integrin, vβ6integrin[190]), reorganization and expression of cytoskeletal proteins[191,192], production of ECM-degrading enzymes, and changes in the expression of specific miRNAs [193,194, 195].

Every day millions of cells are released into the peripheral circulation[196] but if and how an extravasated tumor population grows in a distant organ still an enigma. From all of the steps in the metastatic cascade, the ability to grow in distant organs has the greatest clinical impact. Clinically, many patients treated by local excision of a primary cancer but with micrometastatic disease at the time of diagnosis will show a long latency period before distant disease develops. The ability of the tumor to adapt or to co-opt new growth signals may determine whether distant relapse occurs early or lately.

Moreover, the spread of cancer cells from the primary tumor and the colonization of a distant organ, does not seem to occur in random fashion[197]. Breast tumors usually metastasize to bone, lung, pleura, liver and, less frequently, to brain and the adrenal

gland. ER-positive tumors preferentially spread to bone while ER-negative tumors metastize more aggressively to visceral organs[198,199].

The clinical data for breast cancer has inspired a long-standing debate on whether metastasis follows a traditional progression model or a predetermination paradigm, also known as the Halsted model versus the Fisher model for metastasis[200]. Both models seek to justify and explain clinical data looking at the benefit of aggressive local treatment of the primary tumor and draining lymph nodes versus the early use of adjuvant systemic chemotherapy. Although more anatomic than cellular in nature, the Halsted model looked at breast cancer as a progressive disease that orderly spread pattern from primary site, to regional lymph nodes, to distant organs. This orderly progression would make complete eradication of the primary and regional tumor burden sufficient to stop metastasis. Screening programs, radical versus more limited surgical excisions and the use of adjuvant radiation to the surgical bed can be justified on the basis of the idea that cancers caught early are less likely to spread.

In contrast, Fisher hypothesized that whether distant relapse occurs in breast cancer is predetermined from the onset of tumorigenesis. This view emphasizes breast cancer as a systemic disease and the importance of adjuvant systemic chemotherapy.

The data from randomized trials for adjuvant treatment and from breast cancer screening programs do not clearly rule out one model or the other[201].

To reconcile the clinical data, Hellman[201] proposed that breast cancer is best considered a spectrum of diseases bound by predetermination models and traditional progression models. In fact, in clinical practice, the lack of evidence favors the acceptance of this large spectrum.

II. Dyslipidemia-The problem

As mentioned earlier, breast cancer arises from a combination of genetic and environmental factors; including those from the external environment and the individual-host environment.

The incidence of female breast cancer varies markedly around the world. It is highest in the United States, Western and Northern Europe, intermediate in Southern and Eastern Europe and South America, and lowest in Asia and Africa[202](Figure 6). Since the 1950, breast cancer rates have nearly doubled in traditionally low-risk countries such as Japan[203], Singapore[204], and urban areas of China[205]. Dramatic changes in lifestyle, namely in reproductive behavior and nutritional status occur as these regions make the transition toward a Western-style economy[203,204]. Because of unfavorable trends in these countries, the international gap in breast cancer incidence has narrowed since 1970.

As evidenced by WHO and local databases statistics (Eurostat, CDC), cancer and cardiovascular diseases are the leading causes of death in Europe[2] and USA[1] and are increasing in Asian countries[3,4]. It is reasonable to question if there are some environmental factors responsible for those distributions patterns.

Lipid profile alteration, namely dyslipidemia, is a top risk factor to cardiovascular diseases[206,207, 208]. It is very common in Western countries[209] and is raising in Asia[210], being lipid lowering drugs one of the most used drugs in the world[1].

Dyslipidemia is a broad term that refers to a number of lipid disorders. Most (80%) of them are related to diet and lifestyle, although familial disorders (20%) are also important. The basic categories of dyslipidemias include: elevated low-density lipoprotein cholesterol (LDL-C), low high-density lipoprotein cholesterol (HDL-C), excess lipoprotein(a), hypertriglyceridemia, atherogenic dyslipidemia, and mixed lipid disorders. Most patients with cardiovascular diseases have mixed dyslipidemia (elevated LDL-C and low HDL-C)[211]. In 2008 the global prevalence of raised cholesterol among adults (≥ 5.0 mmol/l) was 39% (37% for males and 40% for females) and had slightly changed between 1980 and 2008. The prevalence of elevated total cholesterol was highest in the WHO Region of Europe (54% for both sexes), followed by the WHO Region of the Americas (48% for both sexes). The WHO African Region and the WHO South East Asian Region showed the lowest percentages (22.6% for AFR and 29.0% for SEAR).

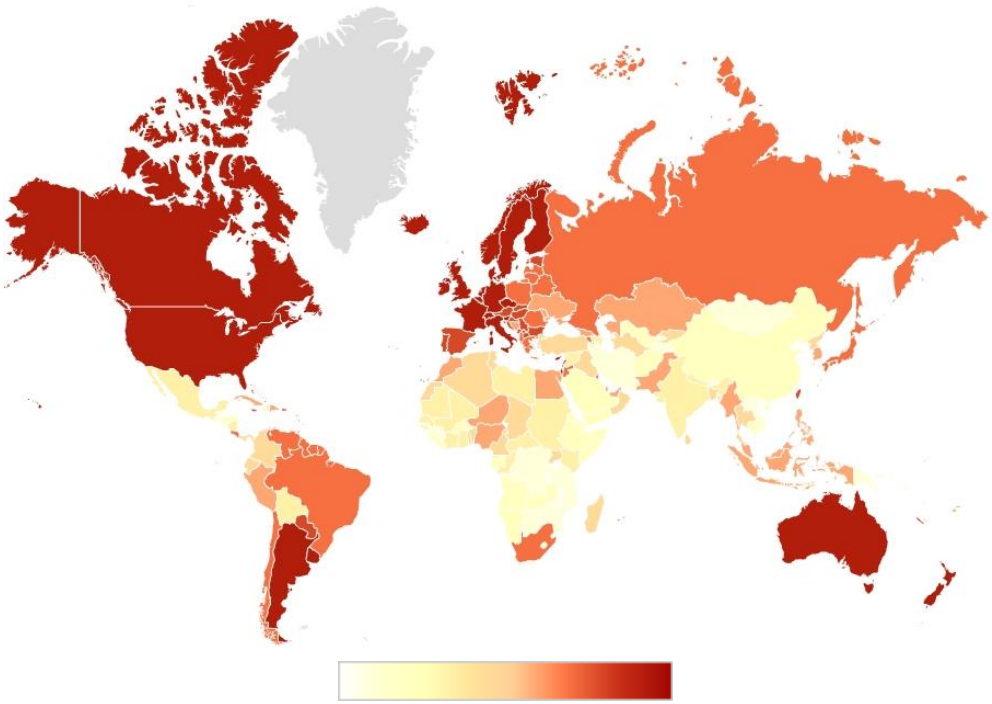


Figure 6: Breast Cancer Incidence, Worldwide in 2008

(Data from Ferlay J et al GLOBOCAN 2008, 2010)

Curiously, the world incidences pattern of raised cholesterol (Figure 7) parallels the breast cancer incidence (Figure 6).

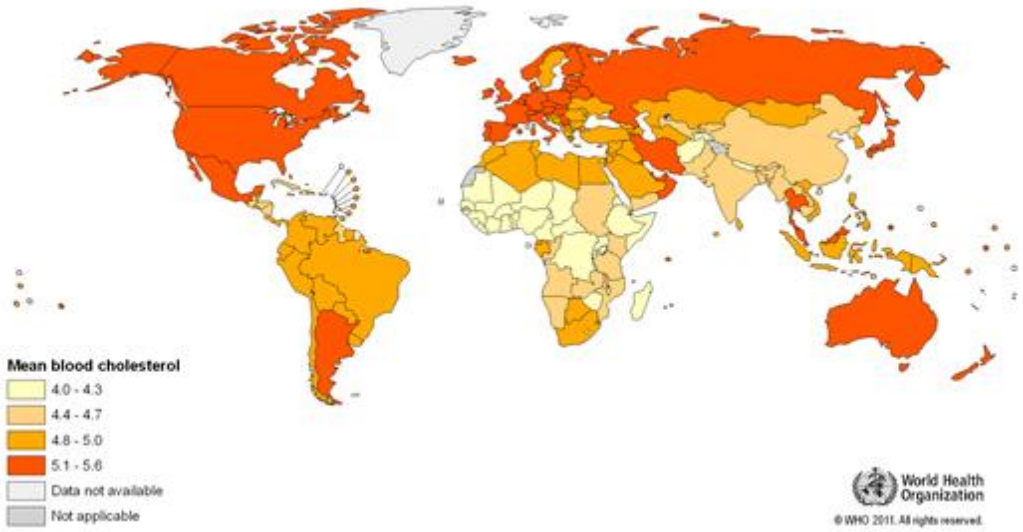


Figure 7: Mean blood cholesterol, ages 25+, age standardized, females 2008

(Data from WHO, 2011)

Overall, raised cholesterol is globally estimated to cause 2.6 million deaths (4.5 per cent of the total number of deaths) and 29.7 million DALYS*, or 2 per cent of total DALYS (*DALY- disability-adjusted life year is a measure of overall disease burden expressed as the number of years lost due to ill-health, disability or early death)[206].

Despite the epidemiologic importance of dyslipidemia, the role of systemic cholesterol metabolism in breast cancer is poorly understood.

Cholesterol and cholesterol metabolism

Cholesterol is a molecule virtually present in all cells and its metabolism is conserved from yeasts to humans, denoting the importance of cholesterol equilibrium to sustain normal functions in eukaryotic cells[212].

Perhaps the most important role of cholesterol is being an essential structural element of the membranes of all eukaryotic cells and subcellular particles. At plasmatic membranes it occupies the spaces between the polar head groups of the phospholipid molecular bilayer, reducing its fluidity and accumulates in microdomains called lipid rafts (for review[213]). In addition to its structural role, cholesterol is an obligatory precursor of steroid hormones (progesterone, estradiol, testosterone, glucocorticoids and mineralocorticoids) and vitamin D[214]. Cholesterol metabolism gives also origin to oxysterols and bile acids (Figure 8).

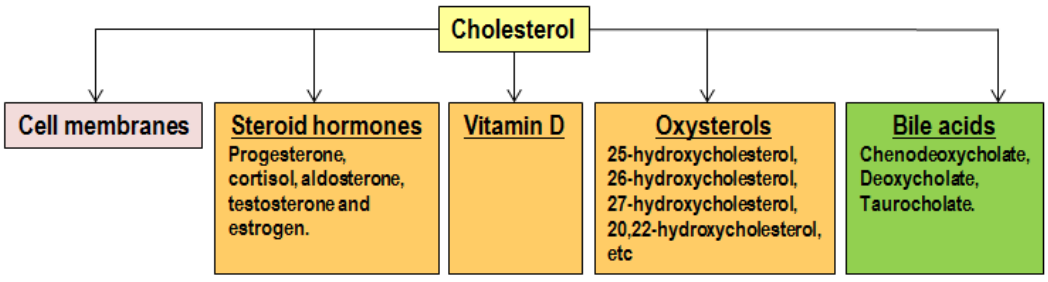


Figure 8: Cholesterol metabolism products.

Cholesterol incorporates cell membranes and its metabolism origins steroid hormones, vitamin D[214], oxysterols and bile acids (Adapted from Yiannis A. et al, 2001[215])

Cholesterol metabolism

The cholesterol molecule is an alicyclic compound and in terms of physical properties, is a lipid with very low solubility in water. For this reason, only about 30% of total plasma cholesterol occurs free; approximately 70% of cholesterol circulates as a component of lipoproteins. In lipoprotein particles the hydrophobic lipid core is surrounded by an amphipathic monolayer of phospholipids and specific apolipoproteins (reviewed by Ginsburg et al[216] and Bachorik et al[217]).

Lipoproteins share common lipid and apolipoprotein components, but apolipoproteins and the amounts of cholesterol, triglyceride, and phospholipids vary between lipoprotein particles. Consequently, lipoproteins can be identified based on molecular mass, chemical composition, physicochemical and flotation characteristics or electrophoretic mobility. The main lipoproteins are commonly classified according to their hydrated densities as follows: chylomicrons (density < 0.95 g/ml), very low-density lipoprotein (VLDL, density 0.95–1.006 g/ml), intermediate-density lipoprotein (IDL, density 1.006–1.019 g/ml), low-density lipoprotein (LDL-C, density 1.019–1.063 g/ml) and high-density lipoprotein (HDL-C, density 1.063–1.210 g/ml). Chylomicrons and VLDL particles are mainly transporting triglycerides, whereas cholesterol is carried by LDL (LDL-C) and HDL (HDL–C) particles.

At birth, serum cholesterol levels are similar throughout the world, typically around 80 mg/dL (2 mmol/ l)[218]. In adults, it spreads in the range of 100-300 mg/dL (2.5–7.5 mmol/L). In many Asian countries, adult levels are often less than 200 mg/ dL (5 mmol/ L), however levels have been raising in the last decades in urban areas, whereas in Europe and the USA they are generally higher than 200 mg/dL (5 mmol/L)[3].

Plasma cholesterol is derived from the diet or can be *de novo* synthesized in the body.

Intestinal cholesterol absorption and the enterohepatic cycle

Cholesterol enters the intestine from diet sources (200-500 mg/day), bile (800–1200 mg/day) and from shed intestinal epithelial cells (300 mg/day). Some 30–60% of intestinal cholesterol is absorbed. Cholesterol can be excreted as fractions of bile salts (250 mg), intestinal cholesterol which are not absorbed (550 mg), and in sebum (100 mg). Nevertheless, a total of some 900 mg must therefore be returned to the liver daily**,** derived from the diet or endogenous synthesis[219].

The absorption and transport of cholesterol in the body involves coordinated combinations of lipoproteins and receptors, as depicted in Figure 9.

Basically cholesterol is absorved from the intestine in chylomicrons and transported to the liver and peripheral tissues in LDL-C. Reverse transportation from peripherical tissues is made mainly by HDL-C.

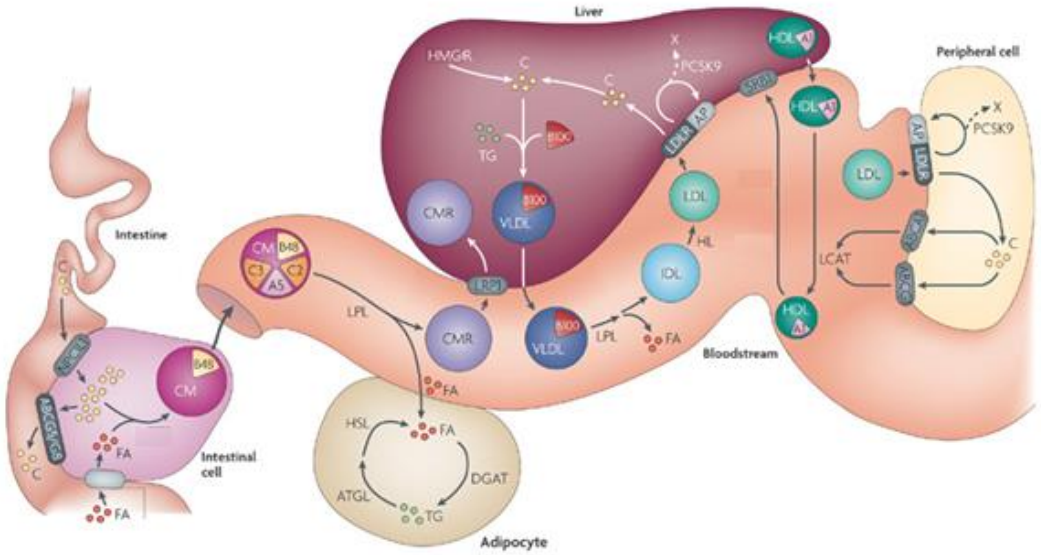


Figure 9: Overview of lipoprotein metabolism

Cholesterol (C) is from dietary is absorved, together with fatty acid (FA), into chylomicrons (CM). CM remnants (CMR) are taken up by hepatic low-density lipoprotein receptor (LDLR), in the absence of LDLR they are taken up by LDLR-related protein-1 (LRP1). In hepatocytes, triglycerides (TG) are packaged with cholesterol and the APOB isoform B100 into very (VLDL); the TG contained in VLDL are hydrolyzed by LPL, releasing FA and VLDL remnants (IDL) that are hydrolyzed by hepatic lipase (HL), thereby yielding LDL-C. In hepatocytes, cholesterol is recycled or synthesized *de novo*, with 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) being rate-limiting. LDL-C transports cholesterol from the liver to the periphery. LDL-C is endocytosed by peripheral cells and hepatocytes by LDLR, assisted by an adaptor protein (AP). Proprotein convertase subtilisin/kexin type 9 (PCSK9), when complexed to LDLR, short-circuits recycling of LDLR from the endosome, leading to its degradation (X). HDL-C, via APOA-I (A1), mediates reverse cholesterol transport by interacting with ATP-binding cassette A1 (ABCA1) and ABCG1 transporters on non-hepatic cells. Lecithin-cholesterol acyltransferase (LCAT) esterifies cholesterol so it can be used in HDL-C cholesterol, which enters hepatocytes via scavenger receptor class B type I (SRB1). (Adapted from Hegele, RA, 2009[220])

Cholesterol synthesis

Cholesterol *de novo* biosynthesis occurs virtually in all cells, although, this capacity is greatest in liver, intestine, adrenal cortex and reproductive tissues.

The cholesterol biosynthesis is carefully regulated. When the amount of dietary cholesterol is reduced, cholesterol synthesis is increased in liver and intestine to supply the needs of other tissues in the form of lipoproteins. When the amount of dietary

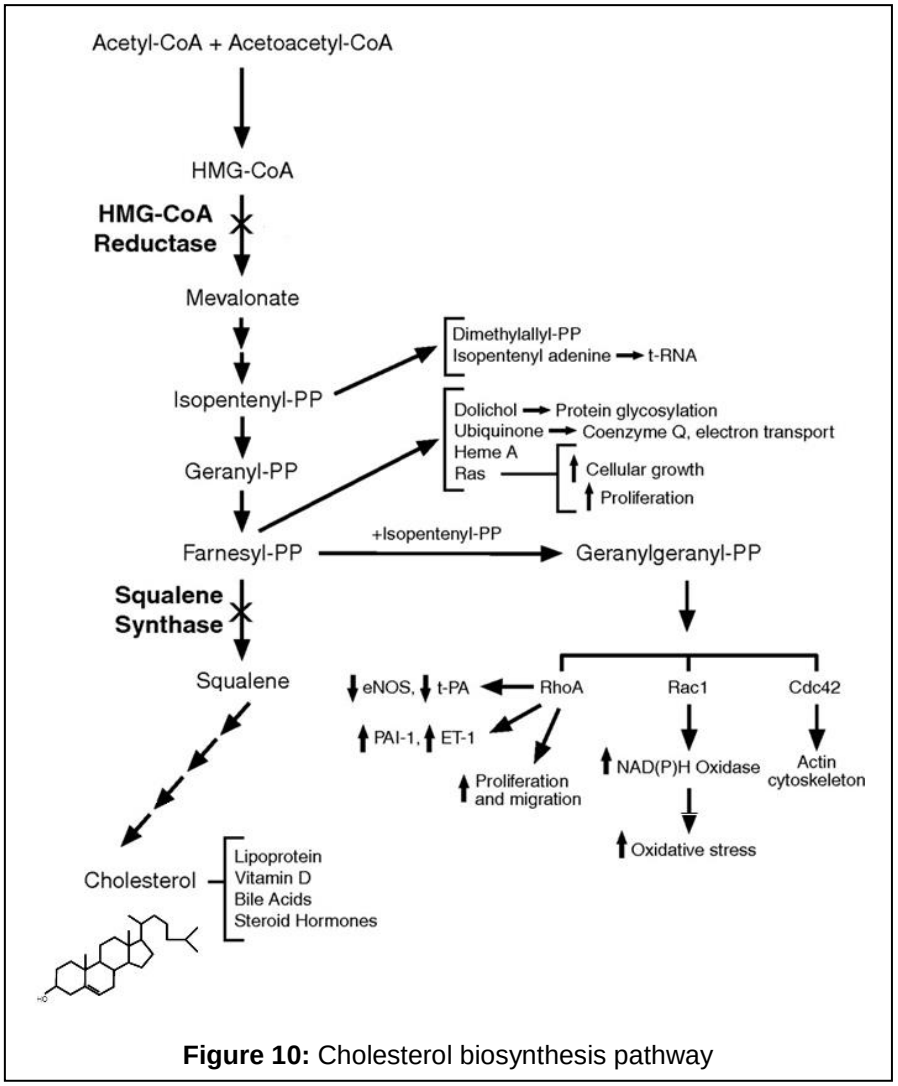


Figure 10: Cholesterol biosynthesis pathway

cholesterol increases, cholesterol synthesis in liver and intestine is almost totally suppressed. Thus, the rate of *de novo* cholesterol synthesis is inversely related to the amount of dietary cholesterol taken up by the body[221]. Hidroxi-3-methyl-glutaril-CoA reductase (HMGR) is located on the RE and catalyzes the conversion of HMG-CoA to mevalonic acid (Figure 10). This is the committed step and the rate-limiting reaction in cholesterol biosynthesis. HMGR is subject to both short-term and long-term control[222]. Long-term effects are mediated by alterations in its rate of synthesis and degradation. Short-term effects involve allosteric effects and alterations in its state of phosphorylation. Squalene synthase catalyses the first commited step in sterol synthesis, since the squalene produced from farnesyl pyrophosphate is converted exclusively into cholesterol and its derivatives (bile acids, vitamin D, steroid hormones), via a complex, multistep pathway[223].

The LDL-C-C receptors

LDL-C receptor is mainly expressed in the liver, but it is also present on the surface of nearly all normal cells where the uptake of plasma LDL-C provides cholesterol for membrane synthesis and other requirements. After binding lipoprotein, the lipoprotein-receptor complex is internalized by endocytosis. LDL-C dissociates from the receptor, which returns to the cell surface and is again able to bind lipoproteins. Within lysosomes,

cholesterol esters of LDL-C are converted to free cholesterol by acid lipase and apolipoproteins are degraded to amino acids. Free cholesterol is delivered to the cytoplasm. Synthesis of LDLR is suppressed when the cell is replete in cholesterol.

The clearance of LDL-C from plasma is primarily mediated by LDLR[224,225]. In addition, some of LDL-C may be removed by LRP1[226], scavenger receptors, which are responsible for the clearance of modified LDL-C [Scavenger Receptors class A (SR-A1/2) and B (SR-BI)][227], ox-LDL receptor (OLR1)[228]), or by non-receptor-mediated internalization[229].

Intracellular cholesterol homeostasis

Despite being a cholesterol-poor organelle, the endoplasmatic reticulum (RE) is the crucial regulatory compartment in cholesterol homeostasis. The RE is the primary site of cholesterol synthesis and esterification, and recent data indicate that excessive free cholesterol may exert its cytotoxic effects via RE perturbation[230].

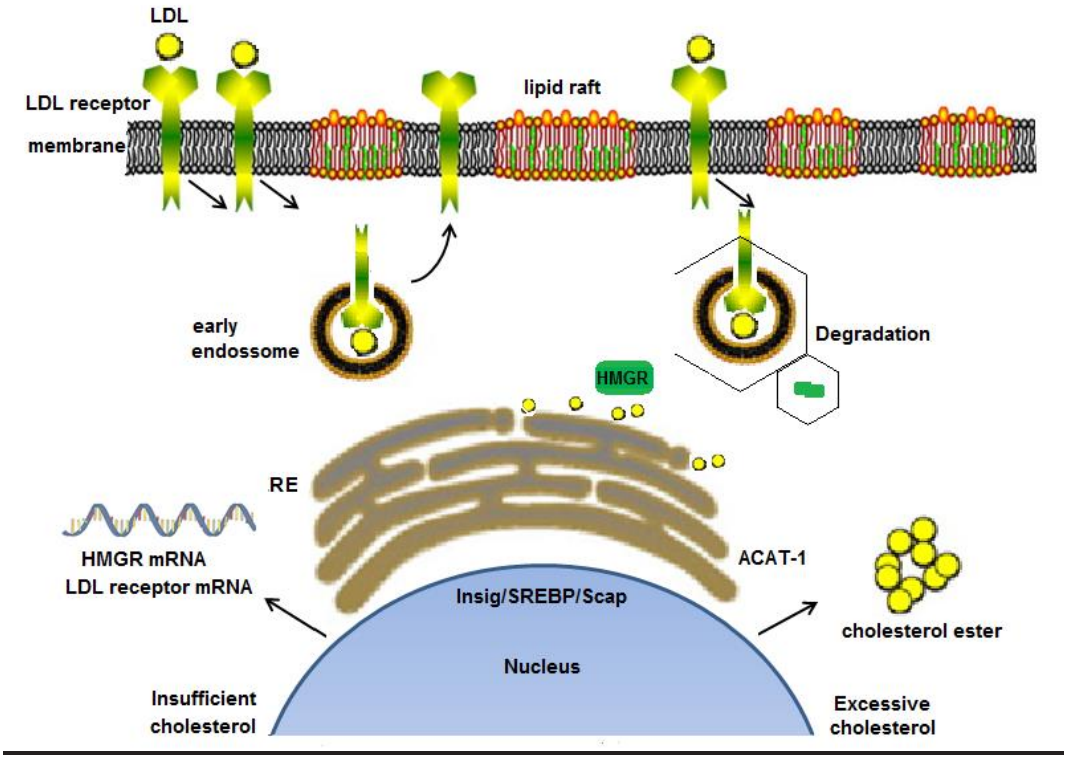


Figure 11: Schematic representation of the intracellular cholesterol homeostasis.

Extracellular cholesterol enters the cell via the LDLR. Transport to the endoplasmic reticulum (RE) occurs via endosomal system. Once at the ER, excess cholesterol is converted to cholesterol ester by ACAT-1. If intracellular cholesterol levels drop, the N-terminus of SREBP is released and enters the cell nucleus, resulting in transcription of mRNA to increase cholesterol production and increase receptors for extracellular cholesterol. If intracellular cholesterol is in excess, HMGR is degraded and LDLR expression down regulated.

To avoid cytotoxicity, free cholesterol is esterified by acetyl-coenzyme A acetyltransferase 1 (ACAT-1) and stored in the cell. When intracellular cholesterol rises, the synthesis is inhibited through degradation of HMGR and the up take is limited by low expression of LDLR. Conversely, when cell requires high levels of cholesterol, its synthesis and up take are up regulated[231](Figure 11).

The homeostatic mechanism whereby cellular cholesterol levels exert negative feedback on cholesterol synthesis is mediated by Sterol Regulatory Element-Binding Protein (SREBP) transcription factors[232]. There are 3 SREBPs: SREBP-2 primarily activates genes involved in cholesterol synthesis, whereas SREBP-1a and SREBP-1c have greater effects on genes involved in fatty acid synthesis. SREBPs are synthesized inactive RE transmembrane proteins. When cholesterol is abundant, SREBPs remain in the RE associated with the escort protein SCAP (SREBP cleavage activating protein) and the RE retention protein Insig[233]. Low cholesterol causes a conformational change in the sterol-sensing domain of SCAP[234], dissociating Insig and allowing SREBP-SCAP to reach the Golgi. Two proteases in the Golgi release the active form of SREBP, which translocates to the nucleus to activate transcription of target genes.

Cholesterol synthesis is also regulated post transcriptionally: high cholesterol accelerates degradation of HMGR, the rate-limiting enzyme in cholesterol synthesis, by promoting association of its sterol-sensing domain with Insig[235]. The final enzyme of cholesterol synthesis, 7-dehydrocholesterol reductase (DHCR7), also has a sterol-sensing domain and may be similarly regulated[236].

Cells with high rates of cholesterol synthesis or accumulation may export cholesterol to cells with increased requirements. The main cholesterol exporters in human cells are ATP-binding cassette protein A1 (ABCA1) and G1 (ABCG1). ABCA1 is responsible for the efflux of both phospholipids and free cholesterol and its transcription is mediated by the nuclear receptors LXRα and LXRβ[237,238,239] whose ligands are sterol metabolites such as 22-, 24-, 27- hydroxycholesterol and 24-25-epoxycholesterol[240,241]. ABCA1 is also post transcriptionally regulated. Several proteins including α1syntrophin, β1syntrophin, calmodulin and apolipoprotein A1 have been reported to interact with ABCA1 and implicated in reduction of the rate of ABCA1 degradation[242,243, 244,245].

In synthesis, the regulation of cholesterol metabolism occurs both at cellular and systemic level. The systemic pool of cholesterol results from the balance between the cholesterol up take from diet, *de novo* synthesis, mainly in the liver, and the adipose tissue storage. The communication is mediated by lipoproteins. The intracellular cholesterol is highly controlled through the coordination of cellular synthesis, up take from the bloodstream,

and storage. This equilibrium involves some key elements such as HMGR, LDLR, ACAT-1 and ABCA1.

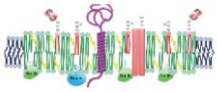
Cholesterol biological functions

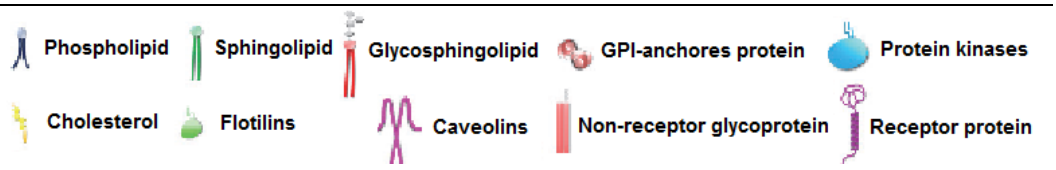
Lipid rafts

Methods to determine the cholesterol content of various cellular membranes are subject to technical limitations, but it is commonly cited that 65% to 80% of total cellular cholesterol is in the plasma membrane, whereas only 0,1% to 2% is in RE[246,247,248]. In the cell membrane, cholesterol is specially localized in sub-domains called lipid rafts[213,249,250].

These microdomains cluster together proteins involved in the regulation of crucial cellular processes[251,252] including cell fate, cell signaling and traffic and cell migratory potential. The structure and function of lipid raft domains depend on their lipid and protein compositions. Two major types of rafts have been distinguished: planar non caveolar and caveolae that form tube-like invaginations of plasma membrane (Table 2).

Table 2: Lipid and protein composition of caveolae and non-caveolae lipid rafts

Lipid Rafts Types	Lipids	Protein Markers	Receptor Proteins	Signaling Proteins
Non-caveolar lipid rafts 	Cholesterol Glycosphingolipid Sphingomyelin Ganglioside GM1 Ganglioside GM3	Flotilin-1 Flotilin-2	Fas EGFR ERBB2 IGF-R1 CD44 ER	Ras Src ERK2 Shc
Caveolae lipid rafts 	Cholesterol Glycosphingolipid Sphingomyelin Ganglioside GM1	Caveolin-1, Caveolin-2 Caveolin-3	Fas EGFR ERBB2 IGF-R1 CD44 ER uPAR MMP-1,2,9	Ras Src eNOS PI3 kinase Phospholipase C



Adapted from Nohara el al[250] and Patra et al[253]. (For legend see Abbreviations.)

Physiologically, lipid rafts contribute to the spatial organization of membrane and, in consequence, to polarization of epithelial cells. This polarization seems to be fundamental to membrane trafficking of cellular components between cell compartments and exchange of cellular components between organelles[254,255]. Lipid raft-mediated trafficking of lipids and proteins facilitates dynamic regulation of cellular signaling cascades[256]. Rafts can also control cell signaling by altering the function of their affiliated proteins. Accumulating evidence suggests that raft associated proteins behave differently whether localized inside or outside of rafts. Modified signal transduction following lipid raft/caveolae disruption has been reported in the case of several signaling cascades involving ERK[257], EGFR[258,259], IGF-R1[260], and platelet-derived growth factor receptor (PDGFR)[261]. Finally, some lipid rafts are actively involved in endocytosis, which promotes internalization of receptors and signaling molecules[262]. Internalization of ligands or receptors modifies downstream signal transduction, and is associated with the perturbation of extracellular ligand-driven signaling events via transient receptor desensitization. Lipid rafts and caveolin-1 have also been shown to be crucial for the formation of invadopodia, membrane protrusions that penetrate the surrounding matrix through a combination of matrix remodeling and physical force[263].

Cholesterol as a progesterone and estrogen precursor

Cholesterol is the precursor of all classes of steroid hormones: glucocorticoids (e.g. cortisol), mineralocorticoids (e.g. aldosterone), and sex hormones (androgen, estrogen, and progesterone). Synthesis and secretion occur in the adrenal cortex (cortisol, aldosterone, and androgens), ovaries and placenta (estrogen and progesterone), and testes (testosterone). Estrogen is also produced peripherally in adipose tissue, which is an important source of estrogen in postmenopausal women

Steroid hormones are transported by the blood from their sites of synthesis to their target organs. Because of their hydrophobicity, they must be complexed with specific steroid-carrier plasma proteins or albumin[264].

Steroid hormones are crucial substances for the proper function of the body. The mineralocorticoids largely function to regulate the excretion of salt and water by kidney. The glucocorticoids affect carbohydrate, protein, and lipid metabolism in manner nearly opposite to that of insulin, and influence a wide variety of other vital functions including inflammatory reactions and the capacity to cope with stress. Both androgen and estrogen affect sexual development and function. They regulate sexual differentiation, the

secondary sex characteristics, and sexual behavior patterns. Progesterone mediates the menstrual cycle and pregnancy events[264].

Steroids can act through two basic mechanisms: genomic and non-genomic. The classical genomic action is mediated by specific intracellular receptors, whereas the primary target for the non-genomic one is the cell membrane. The final hormonal effect in a target tissue is dependent on the cross talk between different nuclear steroid receptors and on expression of receptor isoforms[265].

Oxysterols

Most oxysterols arise from cholesterol by autoxidation or by specific microsomal or mitochondrial oxidations, usually involving cytochrome P450.

Plasma oxysterol levels are increased in hypercholesterolemic humans[266,267], as well as in hypercholesterolemic fed mice[268,269] compared to non-hypercholesterolemic controls. However, reports describing the occurrence and levels of oxysterols in plasma, low-density lipoproteins, various tissues, and food products are hampered by low physiological concentrations (0.01– 0.1mM) relative to cholesterol (5,000mM) and the absence of a comprehensive set of authentic standards.

Oxygenated derivatives of cholesterol (oxysterols) present a remarkably diverse profile of biological activities, including effects on sphingolipid metabolism, platelet aggregation, apoptosis, and protein prenylation. The most notable oxysterol activities center around the regulation of cholesterol homeostasis, which appears to be controlled in part by a complex series of interactions of oxysterol ligands with various receptors, such as the oxysterol binding protein, the cellular nucleic acid binding protein, the sterol regulatory element binding protein (SRBP), the liver X receptor (LXR), and the low-density lipoprotein receptor (LDLR) (for review [270]).

The precise identification of the endogenous oxysterol ligands and elucidation of their metabolism are topics of importance considering their potential physiological role. However, studies of this matter are limited by difficulties in the separation and identification of the various oxysterols and the susceptibility of cholesterol to autoxidation when conserved ex-vivo. This may produces artifactual oxysterols with potent activities when used in experimental studies leading to wrong interpretations of endogenous oxysterols functions[271].

Oxysterols are oxidized derivatives of cholesterol and play various regulatory roles in normal cellular processes such as cholesterol homeostasis by acting as intermediates in cholesterol catabolism.

Cholesterol and cholesterol metabolism in (breast) cancer cells

In normal cells lipogenesis activity is low due to transcriptional regulation of the key genes involved in lipid biosynthesis. By contrast, in tumor cells, increased signaling activity of growth factor or steroid hormone receptors via PI3K/Akt and MAPK/ERK1/2[272], HIF-1, p53[273] and Sonic hedgehog (Shh)[274] pathways modulate and activate SREBP-1, the main regulatory component of lipogenesis. Overexpression of SREBP1 and 2 and lipogenic enzymes was observed in a number of carcinomas, namely breast cancer[275,276] and was described to correlate with disease severity, increased risk of recurrence and lower chance of survival[277]. Also analyses of microarray transcriptional profiling demonstrate an overexpression of cholesterol gene pathways in chemotherapeutic resistant cancer cells[278].

Active sterol biosynthesis is an essential component of proliferating cells since cholesterol is fundamental as a building block for biological membranes. Moreover, the early steps of cholesterol biosynthesis provide cells with compounds essential for cell growth and division, such as mevalonic acid, farnesyl pyrophosphate and geranylgeranyl pyrophosphate important for PI3K, Akt, Ras and other GTPases signaling (Figure 10)[279].

Besides increasing its synthesis, cancer cells can also uptake cholesterol from the bloodstream thought lipoprotein receptors located in the cell surface. High LDLR expression has been shown in breast cancers[280].

However the ability of breast cancer cell to synthetize / up take cholesterol may be diverse[281]. *In vitro* studies using MCF-7 and MDA MB 231 cell lines metabolomics show that ER positive cell lines have up regulation of enzymes from cholesterol biosynthetic pathway, compared to ER negative cell lines[282]. This may underline the estrogen dependency, but also discloses that ER negative cells are more dependent on exogenous than on endogenous cholesterol. In accordance, Wang et al[283], recently showed that genes from lipid metabolism are differently expressed in ER positive and ER negative breast cancers, as well as in contralateral unaffected breast of ER positive and ER negative breast cancer patients. These intriguing findings strongly implicates lipid metabolism in breast cancer initiation and eventually in the generation of different tumor phenotypes.

The promotion of lipogenic and the lipid-consuming behaviors appears to be dependent of proliferating and survival signaling pathways which provide metabolic advantage to cancer cells (Figure 12). However, despite the utility of cholesterol, excessive intracellular accumulation is deleterious[284,285, 286,287].

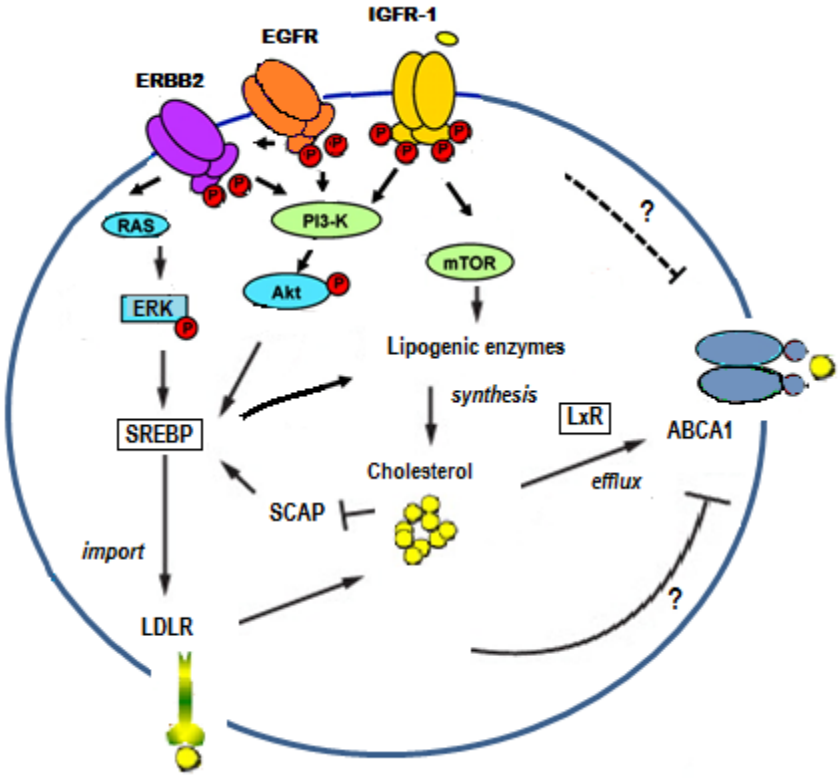


Figure 12: Regulation of cholesterol homeostasis and cancer signaling pathways.

An increased demand for de novo cholesterol biosynthesis is regulated via signaling inputs from cell surface growth factor receptors (e.g. ERBB2, EGFR or IGF-R) that induce transcriptional activity SREBP. Excess of cholesterol will suppress SREBP via its inhibitory interaction with SCAP. The alternative to *de novo* biosynthesis is cholesterol importation via LDLR. LDLR has to undergo lysosomal degradation in order to liberate cholesterol from the endocytosed lipid particles. Oxidized cholesterol species bind to LXR that is a heterodimeric transcriptional factor for various regulatory proteins including cholesterol efflux pumps, ABCA1. How tumor cell regulate intracellular cholesterol content is not completed understood. **(**Adapted from Gorin et al, 2012 [293])

In normal cells, free cholesterol is stored after esterification[288] and the intracellular excess increases expression and translocation of ABC membrane exporters, such as ABCA1 or ABCG1. Loss of function mutations in ABCA1 gene causes Tangier disease and familial hypolipoproteinemia, both cardiovascular conditions characterized by abnormally low levels of HDL-C and atherosclerosis[289] and were recently implicated in cancer cell survival[290]. Several studies suggest that ABCA1 and ABCG1 not only export cholesterol but also generates special membrane meso-domains[291] by promoting lipid redistribution

which could modulate raft dependent cell signaling[291,292,291]. The role of this mechanism in cancer is unknown.

Physiologically, the intracellular cholesterol is regulated by coordination of import/ export machinery (lipoprotein receptors and ABC exporters, as well as HMGR, are under transcriptional influence of SREBP, sensitive to intracellular levels of cholesterol (Figure 12) and also by systemic homeostasis. In cancer cells, is not known how this equilibrium is regulated and simultaneously the metabolic advantage ensured. Furthermore, there is no knowledge how tumor cell metabolism is or not influenced by the systemic cholesterol metabolism.

The elements constituting lipid rafts have been shown to be involved in key processes in cancer cells, namely apoptosis, cell cycle, metabolism and migration.

Abnormal signaling by growth factor receptors can facilitate tumor proliferation and growth. Lipid rafts modulate the signaling functions of several growth factor receptors, including EGFR, ERBB2, ER, IGF-1R and sigma receptors.

EGFR function in particular is largely dependent on its affiliation with lipid rafts[251,294,295,296]. Oncogenic ERBB2 dimerization in breast cancer cells takes place in lipid rafts[297] and forced exclusion of ERBB2 from rafts has been shown to decrease dimerization and tyrosine phosphorylation[297]. Another possible avenue of ERBB2 signaling regulation by lipid rafts relates to protein trafficking. ERBB2 is endocytosed and recycled back to the cell membrane[298] which maintains its overexpression at the cell membrane of breast cancer cells. Modulation of lipid metabolism may control ERBB2 overexpression by influence its endocytosis and redistribution in the cell membrane[299].

Another protein known to localize in lipid rafts is the ER[92]. Estrogen signaling, non-genomic mediated, is linked to lipid rafts, where ER co-localizes with ERBB receptors to modulate growth events[92]. Both these receptors may be activated by membrane-bound ER[300], resulting in MAPK-dependent ER phosphorylation[301]. As these receptors are reportedly lipid raft-affiliated, interference of this union with lipid rafts may prove to be useful in targeting endocrine resistance in breast cancer.

IGF-1R is another tyrosine kinase receptor whose activation leads to proliferation and differentiation via MAPK and PI3K/Akt pathways[302]. IGF-1R activity has been linked to lipid raft affiliation, particularly caveolae. Stable expression of caveolin-1 in MCF7 breast cancer cells, while decreasing cell attachment[303], results in enhanced matrix-independent cell survival via up regulation of IGF-1R and subsequent activation of p53 and p21[304].

Caveolin-1 further drives IGF-1R-induced recruitment of β1-integrin into lipid rafts[305], which could in turn regulate the influence of β1integrin on cell fate[306].

Sigma receptors are a novel family of receptors whose physiological and pathophysiological roles are only beginning to emerge. Their inhibit proliferation, induce apoptosis and can decrease cell adhesion in mammary carcinoma cell lines[307]. Sigma receptors were proposed to have the ability to remodel lipid rafts by modulating raft cholesterol levels via cholesterol-binding motifs[308] and thereby modify lipid rafts signaling.

Defects in apoptosis allow tumor cells to escape growth-inhibitory signals and to progress through the cell cycle. Perhaps the best-characterized death receptor is Fas (CD95 or APO-1), which has been implicated in the apoptotic events that drive physiological remodeling of the mammary gland after breast feeding[309]. Down-regulation of Fas has also been associated with poor prognosis in breast cancer patients[310], and inhibition of Fas activity has been linked to drug resistance[311]. Recent studies have shown that Fas is translocated into lipid rafts, where apoptotic receptor aggregation takes place[312] and cholesterol depletion, in lipid rafts, was shown to abolish apoptosis[313,314]. Acquired resistance of breast cancer cells to Fas-induced apoptosis may alternatively result from activation of survival pathways, such as the PI3K pathway. Its engagement leads to activation of the Akt, which negatively regulates apoptosis by inactivating pro-apoptotic proteins such as Bad and caspase-9[315]. Lipid raft localization of Akt has been implicated in facilitating its activation[316,317].

Cancer cells often activate pathways that reduce adhesion and promote cell migration, increasing the likelihood of the metastatic spread of breast cancer. Kinases play a significant role in regulating cell adhesion and migration. The Src family of kinases (SFK) integrates signal transduction from many tyrosine kinases receptor, including EGFR, IGF-1R and ERBB2[318,319] to multiple downstream targets including PI3K, Ras and FAK[319]. SFK activation has been linked to lipid rafts in breast cancer cells[320], fuelling speculation that selective targeting of raft-affiliated SFK may offer a potent therapy.

As mentioned before, lipid rafts and caveolin-1 are crucial for the formation of invadopodia, by clustering together proteins involved in actin cytoskeleton organization, signaling, cell-ECM adhesion and membrane remodeling[321]. Invasive potential has also been linked with the raft-affiliated proteins caveolin-1 and matrix metalloproteinase 14 (MMP14) in both breast[322] and prostate[323] cancer cells. In fact caveolin-1 and MMP14 have been shown to co-associate and been co-trafficked in invasive breast cancer cell lines[324]. Accordingly, a reduction in ECM degradation activity of MMP14 has been reported in MDA-MB-231 cells following disruption of lipid rafts by cholesterol depletion or

after knockdown of caveolin-1 in MMP14-overexpressiong MDAMB- 231 cells[324]. Together these results highlight that lipid rafts and caveolin-1 are important for invadopodia function in breast cancer cells.

MMP14 is not the only lipid raft-affiliated proteinase implicated in breast cancer progression. Aberrant expression of MMP2 and MMP9, that are localized in rafts during cancer cell migration[253], have been associated with high-grade breast cancer[325].

Similarly, urokinase-type plasminogen activator (uPA) and its receptor (uPAR) have been linked to breast cancer progression and metastasis[253,326], and are also localized to lipid rafts during cancer cell migration[326]. A recent study investigating the importance of lipid rafts in regulating uPAR and MMP9 functionality in breast cancer has demonstrated that cholesterol depletion reduces co-localization of uPAR and MMP9 with lipid rafts and significantly decreases their total protein and mRNA levels[327]. Lipid raft disruption in breast cancer cells resulted in reduced amounts of active Src, FAK, Akt and ERK and increased uPAR co-localization with lysosomal markers, reverted with cholesterol repletion[327]. This is in agreement with previous observations of differences in MMP9-driven cell migration according to its sub-cellular localization inside or outside rafts[325].

A proposed marker for breast cancer stem cells is CD44[328,329] a multi-functional lipid raft-affiliated transmembrane glycoprotein. CD44 is the major receptor for the ECM component hyaluronan[330]; but it can also act as a co-receptor for growth factors[331,332] and organize the actin cytoskeleton through cytoplasmic linker proteins[333]. CD44 abnormalities have been associated with aggressive histological features of breast cancer[334,335], and the association of CD44 with MMP9 in breast tumor cells promotes tumor cell migration and invasion[336].

Thus lipid rafts are fundamental to breast cancer pathogenesis and some current treatments to breast cancer target lipid rafts. One key example is cisplatin, whose mechanism of action is incompletely understood, but which has been described to exert some of its actions through modulation of ceramide lipid rafts. Also some derivatives of doxorubicin, an anthracycline widely used in breast cancer adjuvant chemotherapy, promote their actions by activating lipid raft-associated pathways[337].

Furthermore, lipid rafts are readily modified by diet and nutrition[259,338]. It has been described that *in vivo* polyunsaturated fatty acids (PUFA) supplementation affects lipid raft composition by depleting up to 50% of cholesterol and by altering lipid raft/caveolae protein composition. In comparison to chemical disruption of lipid rafts (e.g. with methyl-β-cyclodextrin and nystatin), PUFA treatment is very selective and depletes only membrane

cholesterol without affecting other cell sources of cholesterol[339]. By this mechanism PUFAs have been shown to decrease cell proliferation and induce apoptotic cell death probably by decreasing Akt/nuclear factor kappa-light-chain-enhancer of activated B (NFκB) signal transduction[340].

Among cholesterol metabolism products, progesterone and estrogen were extensively proved to be breast cancer promoters[32,33]. Both genomic and non-genomic actions have been involved in breast cancer development but also have been important therapeutic targets. Estrogen receptors inhibitors (e.g. tamoxifen) and aromatase inhibitors are extensively used in treatment of patients with ER positive breast cancers and in primary prevention of high risk patients. [35,36,37]

The investigation of the role of oxysterols in cancer is just beginning, but, *in vitro* and *in vivo* studies have suggested that oxysterols may play critical roles in multiple stages of carcinogenesis. First, they may be involved in tumor initiation by enhancing the production of reactive oxygen species/reactive nitrogen species (ROS/RNS). Second, tumor promotion may be enhanced by oxysterols through up regulated expression of proteins such as cyclooxygenase 2 (COX-2) leading to the alteration of cellular features. In addition, certain oxysterols can support cancer progression through the induction of migration. Oxysterols may exert their effect by binding to specific proteins and activating signaling cascades[341]. Very recently, has been shown that 27-hydroxycholesterol increases ER-dependent growth and LXR-dependent metastasis in mouse models of breast cancer linking hypercholesterolemia to breast cancer pathophysiology[342]

Cholesterol and breast cancer

Lipid profile and breast cancer risk

Prospective studies on the relationship between total cholesterol and cancer risk have been limited and contradictory.

Initial studies have reported that cancer incidence[343,344,345,346, 347,348,349,350,351,352,353,354] and cancer mortality[346,350,355,356,357,358,359,354,360,361] were higher in individuals with lower baseline levels of TC. While this inverse association was seen in the majority of the earlier studies, others found higher cancer risk in those with high TC concentrations[362,363,364], nor relation at all [365,366,367,368,369,370,371,372] or U-shaped association, with low as well as high TC levels[373]. Some authors speculated that rather than reflecting a true causal relationship, the higher cancer risk seen for low TC may be attributable to an effect of preclinical cancer. In other words, reflects the metabolic depression of TC due to undiagnosed malignant lesions[347,374]. The positive association of cancer and high blood cholesterol is more consistent from the biological point of view.

To better clarify this issue, the association of cholesterol dietary intake and cancer risk was tested. Hu et al[375] showed a positive relation of high cholesterol consumption and stomach, colon, rectum, pancreas postmenopausal breast, kidney, bladder cancer, non-Hodgkin lymphoma and leukemia.

However, while the majority of these studies are in accordance with these positive associations, some studies did not find any association or revealed contradictory findings. Such as a study[376] described an association of low cholesterol intake and colorectal cancer in the Portuguese population (for review[375]). Recent analysis[377,378] suggested that depending on cancer site, plasma cholesterol levels may have different associations with cancer [379,377].

Concerning breast cancer, prospective studies showed positive association between TC levels and breast cancer incidence[377,363,380] and decreased overall survival[381]. Other studies showed no association[382,379,383,384,385,369], or even inverse correlations between TC levels and incidence of premenopausal breast cancer[348,386,387]; and a protective effect of very high levels of TC[364].

LDL-C and HDL-C are lipoproteins responsible for cholesterol transportation, LDL-C from the liver to peripheral tissues and HDL-C for the reverse transportation[220]. Few studies

specifically addressed the relation of lipoproteins fractions. Regarding HDL-C, some show a positive association between low HDL-C levels and increased breast cancer risk[379,388,389,390,391] and a protective effect of high HDL-C to premenopausal breast cancer[392]. Others authors find no association[393,394,395], and some even report a positive correlation between high HDL-C and breast cancer risk[396,397]. LDL-C is less studied and no association with breast cancer was known[379,384]. Triglycerides levels are not associated with risk in prospective studies except, when in combination with low HDL-C[385,392].

Different study design, study populations and endpoints, duration of follow up, timing of cholesterol measurements, tumor stage and histological type, and differences in statistical adjustment for confounding variables may account for the disparity in the results of these studies.

Lipid profile in breast cancer patients

The lipid profile is the pattern of lipids levels measured in the plasma of a person and usually includes TC, LDL-C, HDL-C and triglycerides. When compared to non-breast cancer patients, alterations in lipid profile are very often seen among breast cancer patients (Table 3

Table 3: Alterations in lipid profile of breast cancer patients

Author, year	Study population	Results*			
		TC	**LDL-C**	**HDL-C**	**TAG**
Bani,1986[398]	N= 48 BC patients N= 50 control	↑ TC	↑ LDL-C	↓HDL-C	↑ TAG
Zielinski,1998[399]	N= 65 BC patients Group I: (N=51) metastatic Group II: (N=14) relapse after remission	Group I: from 32 patients with normal TC at baseline, 32 had ↑ TC with disease progression. Group I: from 9 patients with normal TC at baseline, had 4 ↑ TC with disease progression	NM	NM	Group I: from 28 patients with normal TAG at baseline 22 had TAG ↑ with disease progression. Group I: from 8 patients with normal TC at baseline had 4 TAG ↑ with disease progression
Potischman,1999[400]	N= 83 BC patients (*in situ* 6; Stage I 52; Stage II31; Stage III 20; Stage IV 17) N= 113 control	↓TC advanced stages	NM	NM	NA
Knapp, 1991[401]	N= 83 BC patients (Stage I 20; Stage II11; Stage III 5; Stage IV 47)	↓TC advanced stages vs Stage I; ↓TC bone met vs liver or liver+bone met	↓LDL-C bone met vs liver or liver+bone met	↓HDL-C advanced stages vs Stages I-III	↑ TAG advanced stages vs Stages I-III
Agur-Collins, 1998[402]	N=58 BC patients N= 105 control (afro-american women)	NA	NA	NA	↑ TAG

Ray, 2001[390]	N= 54 BC patients, untreated, Stages II-IV N= 42 control	↑ TC	↑ LDL-C	↓HDL-C	↑ TAG
Hasija, 2005[403]	N= 100 BC patients, untreated Stages I-IV) N= 50 control	↑ TC	↑ LDL-C	NA	NM
Lopez-Saez, 2008[404]	N= 250 BC patients, stages I-III) N= 204 control	↑ TC (BC PM)	↑ LDL-C (BC PM)	↓HDL-C	↑ TAG
Owiredu, 2009[405]	N= 100 BC patients N= 100 control	↑ TC	↑ LDL-C (BC PM)	NA	↑ TAG
Abdelsalam,2012[406]	N= 120 BC patients (Stage I 52; Stage II31; Stage III 20; Stage IV 17) N= 120 control	↑ TC	↑ LDL-C	NA	NM
Yadav, 2012[407]	N= 69 BC patients N= 70 control	↑ TC	↑ LDL-C	↓HDL-C	↑ TAG

* BC patients vs control (when applicable). NM: Not measured. NA: not statistically significant. TC: total cholesterol; LDL-C: low density lipoprotein; HDL-C: High density lipoprotein; TAG: triglycerides; met: metastasis; BC: breast cancer; PM: postmenopausal

The raised TC plasma level is ubiquitous to all studies[390,398,401, 403, 404, 406] with the exception to advanced cases in two studies[400,401]. When measured, triglycerides and LDL-C, were also constantly raised, while HDL-C level was consistently decreased.

The differences observed in breast cancer ptients with tumors at different stages, as well as the modifications in lipid profiles in the Zielinski study[399] underline that the relation of the tumor and cholesterol must be dynamic during the oncologic process.

Furthermore, hormonal therapies used in breast cancer produce changes in lipid profile. Tamoxifen increases triglycerides levels and reduces TC and LDL-C levels[408]. Anatrozole is associated to a non-significant decrease in levels of TC, LDL-C and triglycerides and a significant increase in HDL-C[409]. Letrozole increases significantly TC, LDL-C and HDL-C, with return to baseline after 3 to 6 months[410]. Although considered safe from the cardiovascular point of view, these therapeutic agents can determine changes in lipid profile that may imply uncertain influence in tumor control.

Insights from experimental studies

The influence of plasma cholesterol in breast cancer initiation, from an epidemiological point of view, has been the difficult to demonstrate, whereas controlled experimental studies suggest a role of cholesterol in breast cancer progression.

Since only ER negative breast cancer cell lines display increased proliferation in the presence of LDL-C[411,288], the pro-proliferative and migratory effect of LDL-C appears to be dependent on the status of the ER in breast cancer cell lines.

ER basal like cells store more cholesterol esters that ER positive cells. Acetyl-coenzyme A acetyltransferase 1 is responsible for the storage of long chain fatty acids and cholesterol in cytoplasmatic lipid droplets. Acyl-CoA cholesterol acyltransferase inhibition reduces breast cancer cells proliferation, migration[288], invasion and colony formation[412,413]. Inhibition of intracellular cholesterol transport also affects cellular proliferation[414,415].

The effect of HDL-C on breast cancer cells shows increased proliferation but no migration, invasion or metastasis[416]. On the contrary HDL-C from diabetic patients induced all these effects[417,418,419]. This result is apparently mediated through the scavenger receptor class B, type I (SR-BI)[416,420].

Recent animal studies evidenced a role of high fat diet (HFD) in breast cancer. Llaverias et al[44], used a transgenic mammary tumor mice model (FVB/N mice expressing the polyoma middle T antigen (PyMTTg) under the control of the mouse mammary tumor virus long terminal repeat promoter) to show that HFD mice fed have more aggressive tumors (more tumors, higher histological grade and enhanced angiogenesis) and more lung metastasis. Alikhani et al[421] used an apolipoprotein E (Apo E) deficient mice model. Since Apo E is major ligand to LDLR, these mice developed marked dyslipidemia when challenged with a high fat/ cholesterol diet showing elevated circulating cholesterol and triglyceride levels in the setting of normal glucose homeostasis and insulin sensitivity. Upon injection of two mouse mammary cancer cell lines MET-1 and Mvt-1, the mice developed larger tumors and more pulmonary metastasis than the wild-type mice.

In the study by Kim et al[45], 4-week-old, female BALB/c mice were fed HFD or control diet for 16 weeks. Subsequently, 4T1 mammary carcinoma cells were injected into the inguinal mammary fat pads of mice continuously fed on their respective diets. Results showed that the tumor weight, the number and volume of tumor nodules in the lung and liver as well as tumor-associated mortality were increased in the HFD group.

In all these mice trials, only Llaverias et al[44] measured cholesterol plasma levels. They found that, in animals fed HFD, 12-week-old PyMTTg mice opposed to 4 and 8 weeks old has reduced serum cholesterol levels (P<0.05). As found in some clinical studies this observation is in accordance to the so called reverse causation effect of tumor in cholesterol levels.

In line with these observations, Gomes et al demonstrated that elevated LDL-C promotes bone marrow derived cells (BMDC) mobilization by interfering with SDF-1:CXCR4 axis. These cells are known to play an important role in tumor progression either in the tumor microenvironment[422] or in metastatic niches at distant organs[179].

Very recently, the interest in the role of cholesterol in breast cancer was renewed by the hypothesis that a primary metabolite of cholesterol, oxysterol 27-hydroxycholesterol, promotes ER–positive BC growth in *in vivo* models[342]. In this setting cholesterol reduction would be a safe strategy to prevent and /treat breast cancer[342,423,424,425].

Statins use and breast cancer

HMG-CoA reductase inhibitors (statins) block the rate-limiting step in cholesterol biosynthesis[426]. Statins can be classified, based on their solubility in hydrophilic (better solubility in water) or lipophilic (better solubility in fats) (Table 4). The hydrophilic statins are excreted from the body largely unmetabolized by the liver because hydrophilic nature prevents up take by extra-hepatic tissues. Lipophilic statins are broken down in the liver by the cytochrome P450 (CYP450) system and can be also up take by extra-hepatic tissues[427]. Hydrophilic statins tend to have fewer interactions with other drugs[428].

Table 4: Statins

Classification by solubility	Drug
Water soluble (hydrophilic)	pravastatin, pitavastatin and rosuvastatin
Fat soluble (lipophilic)	atorvastatin, fluvastatin, lovastatin and simvastatin

These drugs have become standard therapy to manage hypercholesterolemia and associated morbidities[429]. As such, statins are among the most commonly prescribed drugs worldwide. Their use has increased dramatically in the past decade and is likely to continue rising[430].

Statins may affect the occurrence or outcomes of diseases either by cholesterol reduction or by mechanisms outside of the cholesterol synthesis pathway[431,432,433]. Pre-clinical studies showed that, contrary to concerns over the carcinogenicity of statins in the early animal models[434], statins may in fact have a chemopreventive potential against cancer[435,436,433].

HMG-CoA reductase is the major rate-limiting enzyme of the mevalonate pathway. Statins inhibition of HMGR prevents the conversion of HMG-CoA to mevalonate, and thereby reduce levels of mevalonate and its downstream products[435]. Many products of the mevalonate pathway are necessary for critical cellular functions such as membrane integrity, cell signaling, protein synthesis, and cell cycle progression[435,436]. Disruptions of these processes in neoplastic cells by statins may result in control of tumor initiation, growth, and metastasis, which has been shown to inhibit cancer cell growth and lead to apoptotic cell death[437,438,439]. Most recently, the possible tumor suppressive activity of statins was linked to down regulation or inhibition of matrix metalloproteinases through prolonged lowering of circulating cholesterol. Metalloproteinases are enzymes able to degrade extracellular matrix components involved in tumor growth, invasion, and metastasis[440].

At the clinical level, the association of statins consumption and cancer risk has been widely searched, across RTC to statins in cardiovascular diseases. Results from two statins randomized control trials, have related an increase incidence of cancer risk with pravastatin use[441,442], but no other RTC or meta-analyses confirmed such effects and showed a neutral impact of statins[443, 444,445,446,447,448,449,450,451].

Importantly, those studies were designed to assess cardiovascular endpoints and therefore enclose many limitations to assess cancer risk. Relatively short follow-up times, short duration of statin use, no evaluation of dose-duration response, highly selected groups of patients in RCTs, and failure to account for multiple types of statins must be considered when interpreting results of all the meta-analyses. Mean follow-up in statin RCTs is generally 4–6 years and cancer is an endpoint that needs longer follow-up. Meta-analyses of overall cancer risk are unlikely to be very sensitive as it is unlikely that statins alter the risk of all cancers. A true change in the risk of specific cancers may be masked

by the random variation in the association of statins with other cancers. Some authors also suggested that different pharmacokinetic properties of hydrophilic statins (e.g. pravastatin) versus hydrophobic statins (e.g. lovastatin, simvastatin) support opposing effects on cancer risk[452,453]. This may explain the increased risk of cancer found among pravastatin users in two of the large RCT[441,442], and the protective effect seen in users of lipophilic statins in other studies[454,455,456]. However, the two published meta-analyses reported on statin type found no difference in risk by hydrophilic versus hydrophobic statins[450,446].

A very recent cohort study crossing statins prescription/ pharmacy dispense and cancer related-mortality in Denmark National databases[457], showed that statins use before cancer diagnosis is associated with reduced cancer-related mortality. Although some limitations found and pointed by the authors and others[458], such as lack of follow up and co-morbidities data, the association seems to be plausible, in the study population.

Concerning breast cancer, most studies[383,459,460,461,462,463,464,465,466,467,468,469,470], including meta-analysis[471,450,446,472,473,474] have reported no association between breast cancer incidence and statin use, although some reports suggested protective effect[475,476,456,455,477,478] and one an inverse effect[479] (Table 5).

Two studies analyzed the association between post-diagnosis statin use and breast cancer recurrence. Kwan et al[480] followed a cohort of approximately 2000 breast cancer survivors for a mean of 5 years and observed an association between lipophilic statin use and decreased cancer recurrence. While in this study the estimate was not measured precisely enough to provide strong evidence against a null association (hazard ratio [HR] = 0.67, 95% confidence interval [CI] = 0.39 to 1.13), Ahern et al[481] following a large Danish cohort of stage I-III breast cancer patients diagnosed between 1996-2003, found that simvastatin use (but not hydrophilic statins) was associated with reduced breast cancer recurrence (10 y adjusted HR 0,55 96CI 0,35-0,85). Others have found a protective effect higher in ER negative breast cancer[478,482] and a putative potentiation of radiotherapy effect[483,484,485].

While there are many observational studies and meta-analyses published on statin use and breast cancer risk, the current data is unsatisfactory for recommending statins for primary breast cancer prevention and secondary prevention is not well studied.

Table 5: Epidemiologic studies on the association of statins use and breast cancer risk.

Author, year	Design*	Number of cases		Results
		User	Non-user	(point estimate (95% CI))**
Blais, 2000[459]	CC	65		OR=0,67 (0,33–1,38)
Coogan, 2002[479]	CC	33	828	OR=1,5 (1,0–2,3)
Kaye, 2002[460]	CC	31	102	OR=1,0 (0,6–1,6)
Beck, 2003[476]	Cohort	188	691	Ever use: OR=1,09 (0,93–1,28) Age <55 yrs: OR=0,81 (0,53–1,24) Age >55 yrs: OR=1,15 (0,97–1,37) Use ≥4 yrs: OR=0,26 (0,12–0,55)
Cauley, 2003[475]	Cohort	6	234	RR=0,28 (0,09–0,86)
Boudreau, 2004[455]	CC	112	849	Ever use: OR=0,9 (0,7–1,2) Use> 5 yrs: OR=0,7 (0,4–1,0)
Graaf, 2004[461]	CC	467		OR=1,07 (0,65–1,74)
Kaye, 2004[462]	CC	40		OR=0,9 (0,6–1,3)
Kochhar, 2005[477]	CC	556		OR=0,49 (0,38–0,62)
Eliassen, 2005[383]	Cohort	152	1,472	Current use: RR=0,91 (0,76–1,08)
Friis, 2005[463]	Cohort	48	3,093	RR=1,02 (0,76–1,36)
Cauley, 2006[456]	Cohort	297	4,086	Ever use: HR=0,91 (0,80–1,05) Hydrophobic statin use: HR=0,82 (0,70-0,97)
Setoguchi, 2007[464]	Cohort	203	65	HR=0,99 (0,74–1,33)
Boudreau, 2007[467]	Cohort	130	2,577	Ever use: HR=1,07 (0,88–1,29) Use ≥5 yrs: HR=1,27 (0,89–1,81) Hydrophobic use: HR=1,01 (0,80–1,26)
Coogan, 2007[465]	CC	69 cases and controls	1101 cases and controls	Ever use: OR=1,2 (0,8–1,8) Use ≤1 year: OR=1,0 (0,5–2,1) Use 1–5 year: OR=1,2 (0,7–2,1) Use ≥5 years: OR=1,5 (0,7–3,2) P for trend in duration: 0,1 Hydrophobic statin use: OR=1,0 (0,6–1,6)
Friedman, 2007[468]	Cohort	881	-	Ever use: HR=0, 9 (0,92–1,06) Use >5 yrs: HR=1,02 (0,86–1,21)
Smeeth, 2008[469]	Cohort	324	2880	HR=1,17 (0,95–1,43)
Kumar, 2008[478]	Cohort	387	1754	Use ≥ 1 yrs ER-: HR 0,63 (0,43-0,92) Use <1yr ER+: HR 1,25 (0,72-2,17)
Haukka, 2010[470]	Cohort	-	-	RR=1,01 (0,96–1,06)
Woditscha, 2010[466]	CC	5,409	17,079	Lipophilic statins Use ≥ 2 yrs ER-: HR 0,98 (0,84-1,13) Use≥ 2 yrs ER+: HR 1,03 (0,97-1,10)
Kawn , 2008[480]***	Cohort	367	1444	RR = 0.67; (0.39-1.13) P linear for trend in duration: 0,02
Ahern, 2011[481]***	Cohort	3282	15 487	Hydrophobic statin use HR: 0,55 (0,35-0,85)

*CC=case control; **OR=odds ratio; RR= relative risk; HR=hazard ratio; CI=confidence interval; relative to non-users; *** recurrence risk.

Of the 27 ongoing studies registered in ClinicalTrials.gov examining associations between statin therapy and cancer risk or outcomes, nine are in breast cancer. Results of these ongoing trials will be a major contribution to the field. Such trials and epidemiologic studies are more feasible for cancer prognosis than incident risk due to the long latency period of cancer and thus, the long follow-up periods required. However, the prospect of reducing the incidence and burden of some of the most prevalent cancers with a safe, affordable, and tolerable medication that already reduces the risk of the leading cause of death, cardiovascular disease, warrants further exploration.

Non-statin cholesterol lowering drugs are a heterogeneous group with varied mechanisms of action including fibrates, niacin, bile-acid resins and cholesterol absorption inhibitors. Few studies evaluated other cholesterol-lowering drugs and, in general, they are not associated with risk of cancer[486] except cholestyramine, and fibrates[434] in older animal studies, but human association was not demonstrated.

Hypothesis and Aims

Based on epidemiological and experimental data we considered the hypothesis that systemic cholesterol metabolism influences breast cancer behavior by promoting tumor progression.

To test our hypothesis we sought to:

1) Determine an association of systemic cholesterol levels and breast cancer progression.
   For that we designed a clinical prospective study of women with early breast cancer to determine how lipid profile correlates with tumor features and progression. We also tested the hypothesis experimentally by using orthotopic breast cancer mouse models and *in vitro* breast cancer cell lines.

2) Investigate the cellular and molecular mechanisms by which cholesterol can influence breast cancer pathophysiology.

3) Explore how assessment and control of the systemic cholesterol levels would improve breast cancer treatment and surveillance.

Chapter 1

Plasma Level of LDL-Cholesterol is a Predictive Factor of Breast Tumor Aggressiveness[1]

Introduction

Cancer and cardiovascular diseases are the leading causes of death in Europe[2] and USA[1] and their incidence is also increasing in Asia[3,4].

Breast cancer is the most frequent malignancy diagnosed each year in Europe and USA (age–adjusted incidence rate, 76-89,7 per 100,000)[202], and still account for high mortality rate[3]. In Asia, incidence and mortality of breast cancer are lower (age–adjusted incidence rate, 22-30 per 100,000)[202] but have been dramatically raising in the last decade[3, 205].

Lifestyle and diet are frequently indicated as reasons for the global distribution of breast cancer incidence[38,39]. Nevertheless, while hypercholesterolemia, mainly attributed to diet [high LDL-C and low HDL-C levels] was already shown to play a major role in the etiopathogenesis and incidence of cardiovascular diseases[211], its influence in breast cancer is not completely understood.

Several reasons suggest the possible involvement of cholesterol in breast cancer biology. Cholesterol is a structural component of cell membrane, specially localized in lipid rafts-membrane microdomains that assemble the machinery of cell signaling pathways closely associate with malignant transformation due to their influence in organization of the cytoskeleton, cell polarity and angiogenesis[256,487]. Cholesterol is also a steroid hormone

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1 The results discussed in this chapter are published in Rodrigues Dos Santos C, Fonseca I, Dias S, Mendes de Almeida JC**. Plasma level of LDL-cholesterol at diagnosis is a predictor factor of breast tumor progression.** *BMC Cancer*. 2014 Feb 26;14:132 (highly accessed paper), and were presented at 34th Annual San Antonio Breast Cancer Symposium, 13th Annual Meeting of the American Society of Breast Surgeons and VIII Congresso Nacional de Senelogia.

Preliminary results of this study were published at Santos, CR; Mendes Almeida JC and Dias, S, **Systemic LDL Promotes Breast Cancer Progression;** *Annals of Surgical Oncology*, May 2012, Volume 19 (Issue 2 Supplement);100-01.

precursor, namely estrogen and progesterone, and the vast majority of breast cancer is hormone responsive[488]. Moreover, the peak incidence of breast cancer occurs in the perimenopausal period[489], when women dyslipidemia prevalence also increases[490].

However, clinical data searching for the association of cholesterol levels and breast cancer risk are scarce and have provided contradictory results. Prospective studies showed positive association between total cholesterol levels and both breast cancer incidence[377,363,380] and overall mortality in breast cancer patients[381]; but also no association at all [382,379,383,384,369,385] or even inverse association between total cholesterol levels and incidence of premenopausal breast cancer[348,386,387]; as well as a protective effect of very high levels of total cholesterol[364] were described. Studies specifically addressing the role of lipoproteins fractions are similarly contradictory. Regarding HDL-C, some shown a positive association between low HDL-C levels and breast cancer risk[379,388,389,390,391] and a protective effect of high HDL-C with premenopausal breast cancer[392]; while others found no association[393,394,395], and some even reported a positive correlation between high HDL-C and breast cancer risk[396,397]. LDL-C is less studied and no association with breast cancer was reported[379,384]. Triglycerides levels were no associated with risk in prospective studies except, in combination with low HDL-C[385,392].

Disparity in those results may be due to different study designs, study populations, endpoints, duration of follow up, timing of cholesterol measurements, tumor stage, tumor histological type, and differences in statistical adjustment for confounding variables.

On the other hand, alterations in lipid profile are often seen among breast cancer patients, when compared to non-cancer controls. Increased total cholesterol levels were transversally seen in almost all studies[390,398,403,404,406,405,407.400,401]. Triglycerides and LDL-C, when measured, were also constantly elevated while HDL-C level was decreased [390,398,403,404,406,405,407,402]. Experimentally, lipoproteins were shown to induce cancer cells proliferation and migration *in vitro*[411,288,416,491,417,418,419] and studies using genetic or diet induced hypercholesterolemic mouse models revealed a clear association between high lipid levels and breast cancer development[44] and progression[45,421].

Thus, whereas the influence of plasma cholesterol in breast cancer risk has been difficult to demonstrate, the variations of lipid profile in breast cancer patients and the results from controlled experimental studies strongly support a role for cholesterol in breast cancer pathophysiology.

At this point we hypothesized that host cholesterol-enriched systemic environment promotes breast tumor progression. To answer to this question we analyzed the

correlation of lipid profile with clinical and pathological characteristics of breast tumors in a cohort of breast cancer patients without previous treatment.

Methods[2]

Study population and data collection

From January to December 2011, women, who underwent for operable breast cancer at the Breast Unit of Instituto Português de Oncologia de Lisboa, Francisco Gentil (IPOLFG), were prospectively assembled (Figure 13).

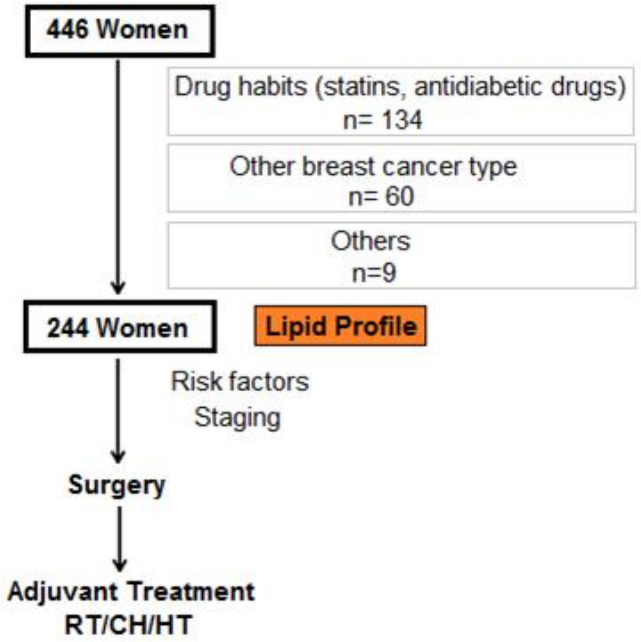


Figure 13: Study Fluxogram

Biospecimen collection and plasma lipid and lipoproteins assays

Fasting lipid profile was measured at diagnosis, along with routine preoperative exams. Blood was collected into EDTA-coated tubes and the plasma levels of TC, LDL-C, HDL-C and triglycerides were measured automatically by electrophoresis (Architect ci8200 analyzer; Abbott Diagnostics, Wiesbaden, Germany) at the certified Clinical Pathology Laboratory of IPOLFG.

Hormonal receptors were measured using standardized immunohistochemistry. HER2 was scored according to the WHO guidelines[493] from 0 to 3+. All cases with 2+ score were

[2] The data collection was performed in collaboration with Breast Unit clinicians. Immunohistochemistry was performed by Pathological Department technicians and reviewed by Isabel Fonseca. Plasma lipids were measured by Clinical Pathology technicians.Assistance with statistical analysis was obtained from Nuno Cortez-Dias. The remainder tasks described in this chapter were performed by the candidate.

reevaluated using chromogenic *in situ* hybridation. Immunohistochemical staining for Ki67 was performed in a Dako Autostainer® (Dako, Glostrup, Denmark) using standard protocols, followed by counting positive cells in an automated cellular imaging system (ACIS®II, Dako, Glostrup, Denmark I). Technicians were blinded to the lipid profile status of the study participants.

Statistical analysis

Continuous variables are presented as mean (standard deviation) or median (interquartile range) if they have normal distribution or not, respectively. For categorical variables absolute values and frequencies are shown. Spearman rank correlations coefficients were calculated to examine correlations between continuous variables. Univariate analysis between lipid profile, breast cancer risk factors (menopausal status, BMI, age, family history, parity, breast feeding) and traditional prognostic factors (tumor size, positive lymph nodes, tumor grade, lymphovascular invasion, ER, PR, HER2 and Ki67) were performed using parametric tests to variables with normal distribution and non-parametric tests to variables without normal distribution. Patient subgroups were defined by LDL-C levels tertiles. Tumor characteristics (T stage, N Stage, immunohistochemical subtypes) were studied as categorical variables.

Multivariate logistic regression to the risk of tumor T stage included the following variables: TC (by tertiles), LDL-C (by tertiles), triglycerides (3rd tertile), BMI (by tertiles) and age (by tertiles) by using stepwise conditional forward analysis.

Likelihood ratio *P* values are reported to whole variables in the model. All *P* values are two-tailed. The statistical analysis was done using IBM SPSS Statistics for Windows, Version 19.0(Armonk, NY: IBM Corp. Released 2010).

Results

A total of 446 women were potential assembled to the study (Figure 13). Of those, 202 were excluded for being on conflicting medications (n=134), because histological type (n=60) and previous treatments (n=9). Baseline demographic, clinical and tumor related characteristics of the study population (n=244) are listed in (Table 6).

Table 6: Clinical and tumor-related characteristics of the study population (N=244)

Characteristics		No. of Patients	%[1]
Patient Characteristics			
Age (Years), mean±SD (range)	58,2±13,3 (29-91)		
Weight (Kg), median (interquartile range)	67 (60-76)		
Height (cm), median (interquartile range)	160 (154-163)		
BMI (Kg/m^2), median (interquartile range)	26,7 (23,5-30,44)		
Menopausal Status (yes)		126	65,6
Gestation (yes)		182	90,5
Breast-Feeding (yes)		146	79,3
Oral Contraception/ HT (yes)		96	55,6
Family History of BC♯(yes)		56	27,5
TC (mg/dL), median (interquartile range)	209,5 (191-231)		
HDL-C (mg/dL), median (interquartile	53 (46-60)		
LDL-C (mg/dL), median (interquartile	128 (110-153)		
Triglycerides (mg/dL), median (interquartile range)	94 (74,5-126)		
Tumor Characteristics			
Histological Type	IDC[2]	244	100
Tumor Size (mm), median (interquartile range)	21 (14-30)		
Tumor Stage (T)	**T1, ≤ 2cm**	122	50
	T2, 2-5cm	115	47,2
	T3, >5cm	7	2,8
Tumor Grade	**G1**	27	12,4
	G2	134	61,5
	G3	57	26,2
Subtypes	**ER/PR positive∞**	204	83,9
	Triple negative	23	9,5
	HER 2	16	6,6
LVI positive		61	29,2
Nodal Stage (N)	**N0**	137	56,9
	N1, 1-3 LN+	72	29,9
	N2, 4-9 LN+	18	7,47
	N3, ≥10 LN+	14	5,8
Clinical Stage	**I**	84	34,4
	II	125	51,3
	III	35	14,3
Bilaterality (yes)		10	4,1

[1] frequency of known cases; [2] IDC is presently named invasive carcinoma, NOS.∞ includes Luminal A and B. HT: Hormonal Therapy; BC: Breast Cancer; ♯ not in the first generation; BMI: Body Mass Index; HT: Hormonal therapy; TC: Total Cholesterol; LDL-C: Low Density Lipoprotein; HDL-C: High Density Lipoprotein IDC: Invasive Ductal Carcinoma; ER: Estrogen Receptor; PR: Progesterone Receptor; LVI: Lymphovascular Invasion; LN: Lymph Nodes.

Spearman correlations

Exploratory correlations between lipid profile, age, BMI, primary tumor size and lymph node metastasis ratio (defined as the number of metastatic axillary lymph nodes over the total lymph nodes removed), showed that systemic levels of LDL-C and TC correlates positively with tumor size (Spearman r=0,199, *P* 0,002; Spearman r=0,145, *P* 0,025, respectively). As expected, age correlates with BMI (Spearman r= 0,155, *P* 0,022) and triglycerides (Spearman r= 0,312, *P* <0, 0001) and BMI correlates with age, LDL-C (Spearman r=0,161, *P* 0,018), HDL (Spearman r=-0,157, *P* 0,021) and triglycerides (Spearman r=0,149, *P* 0,027).

Plasma LDL-C level was significantly related to tumor T stage and prognostic groups of the AJCC[78]. There is no statistical difference in other parameters of lipid profile across tumor stages (Table 7).

Table 7: Lipid profile in tumor stage[1] and in prognostic groups[2]

Stage/ Level (Median, interquartile range)	**Tumor Stage T1 (≤ 2cm)**	**Tumor Stage T2 (2-5cm)**	**Tumor Stage T3 (> 5cm)**	***P* value*[1]**	**Stage I**	**Stage II-III**	***P* value*[2]**
TC level, mg/dl	205 (183-228)	212 (191-234)	196 (189-225,5)	0,105	202 (179,5-227)	210 (194-233)	0,053
HDL-C level, mg/dl	53 (48-62)	52 (47-60)	51,5 (45,5-53,8)	0,229	53 (46-62)	52,5 (46,3-60)	0,566
LDL-C level, mg/dl	125 (100-148)	131 (109,5-156)	138 (118-159,5)	0,015	124 (96,5-147)	130,5 (114-153)	0,013
TAG level, mg/dl	89 (75,5-117,5)	104 (75-132)	100 (72,5-115)	0,212	92 (77,3-118,8)	97 (74,5-129,5)	0,512
LDL-C/ HDL-C	2,3 (1,7-3,1)	2,6 (1,9-3,1)	2,3 (2,4-2,8)	0,020	2,3 (1,7-3,1)	2,6 (2,0-3,1)	0,040

[1] TNM Classification. [2] The American Joint Committee on Cancer (AJCC) Staging System [*1] Kruskall-Wallis Test, [*2] Mann-Whitney Test. TC: total cholesterol; LDL-C: Low Density Lipoprotein; HDL-C: High Density Lipoprotein; TAG: triglycerides

Univariate associations

Population was stratified based on LDL-C level tertiles: LDL T1:LDL-C≤117mg/dl; LDL T2:144mg/dl≥LDL-C>117mg/dl; LDL T3:LDL-C>144mg/dl. Patients in the third LDL-C tertile have larger tumors (*P* 0,024) (Figure 14A), of higher differentiation grade (*P* 0,027), with higher proliferative rate (*P* 0,017), and higher likelihood of being diagnosed in advanced stages. This analysis does not demonstrated differences in lipid profiles between breast tumor immunohistochemical subtypes (Luminal A, Luminal B, Triple negative and HER2 type[494]). Nevertheless, tumors of patients in the third LDL-C tertile are more commonly HER2 positive, when compared to the others tertiles (*P* 0,002) (Figure 14B). There are no differences, between LDL-C categories, concerning studied breast cancer risk factors (Table 8).

Table 8: Patient characteristics in LDL-C levels tertiles

Patient Characteristic	**LDL T1 (LDL≤117mg/dl) N=82**	**LDL T2 (144mg/dl≥ LDL>117mg/dl) N=81**	**LDL T3 (LDL>144mg/dl) N= 81**	***P* value**
Age (years), median (interquartile range)	60,14 (49,0-70,3)	58,3 (48,2-70,0)	58,2(50,9-64,2)	0,593
Menopausal Status (+), (N,%)	64,7%	65,1%	67,2%	0,951
Pregnancy History (+), (N,%)	94,2%	71,9%	88,3%	0,408
Breast –feeding (+), (N,%)	76,6%	51,9%	68,4%	0,079
Oral contraception (+), / HT (+), (N,%)	51,6%	58,3%	53,7%	0,597
Family History♯, (N,%)	40%	34,5%	25,5%	0,258
BMI (Kg/m^2), median (interquartile range)	25,7(22,8-29,4)	26,1(23,4-29,4)	27,9(24,7-31,5)	0,107

HT: Hormonal Therapy; BC: Breast Cancer; BMI: Body Mass Index, ♯ not in the first generation. *P* value: Kruskall-Wallis test. LDL:LDL-C: Low Density Lipoprotein ; BMI: body mass index; T: tertile level.

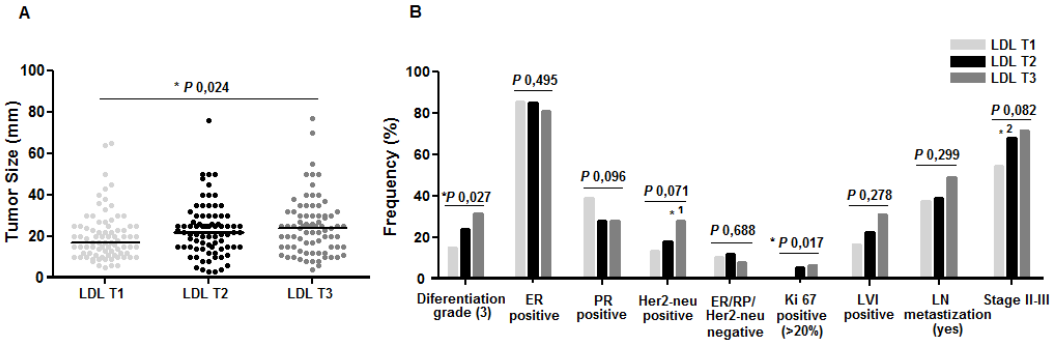


Figure 14: Tumor characteristics-LDL tertiles

A. Tumor size increases across LDL-C tertiles groups. Line represents the median value of tumor size in each LDL-C tertile. **B**. Frequency of tumor characteristics in LDL-C tertiles groups. [*1] LDL T1-T2 (Her2-neu+ 15,5%) vs LDL T3 (Her2-neu+ 27,8%): OR 5,015 (1,678-14,988) *P* 0,002 . [*2] LDL T1 (Stage II-III 54,9%) vs LDL T2-T3 (Stage II-III 69,6%): OR 0,543 (0,313-0,943) *P* 0,029. Kruskall-Wallis test. ER: Estrogen Receptor; PR: Progesterone Receptor; LVI: Lymphovascular Invasion; LN: Lymph Nodes.

Multivariate logistic regression

A multivariate logistic regression to the risk of tumor T stage was then modeled. All the variables significantly associated at univariate analysis (Additional Table 1) were introduced, including age and BMI, even not positively associated, because of its strong correlation with lipid profile. It was found that the LDL-C level higher than 117mg/dL is a predictor factor to tumor size ≥ 20mm, at diagnosis (Table 9).

Table 9: Univariate and multivariate logistic regression to the risk of tumor size ≥20mm.

Variable	*Univariate Analysis*			*Multivariate Analysis*		
	HR	*95% CI*	*P value*	*HR*	*95% CI*	*P value*
Total Cholesterol T≥2vs T1	1,912	1,113-3,285	0,018			
LDL-C (>117mg/dl) T≥2vs	2,419	1,394-4,199	0,002	**2,468**	**1,356-4,491**	**0,003**
Triglycerides T3	1,888	1,092-3,264	0,022			
BMI T≥2vs T1	1,785	0,010-3,155	0,045			
Age T≥2vs T1	0,833	0,430-1,416	0,499			

LDL-C: Low Density Lipoprotein; BMI: body mass index; HR: hazard ratio, CI: confidence interval

Discussion

Multiple epidemiological studies exploring causal associations between dyslipidemia and breast cancer incidence produced contradictory results[382,379,383,384,385,348,386,387,377,363,364,369,380.] Several methodological aspects may explain the diverse conclusions, but biological clues from laboratory[411,288,416,491,417,418,419] and *in vivo* pre-clinical studies[45,342,44,421], as well as, significant alterations in lipid profile of breast cancer patients compared to healthy controls[390,398,403,404,406,405,407,400,401,374,495,390,398,403,404,406,405,407,402] are very suggestive of a role for cholesterol in breast cancer progression.

In the present study, fasting lipid profile (with discrimination between lipids and lipoproteins fractions) was prospectively assessed in a cohort of patients with invasive carcinoma, in early stage, before any treatment and with no history of being on lipid lowering drugs (including statins, fibrates, oral anti-diabetics, insulin or corticosteroids). Population characteristics are similar to other series, concerning to demographic and tumor characteristics[496,497,498]. Slight under-representation of triple negative and HER2 type cancer have occurred due to the inclusion criteria, as those patients are more likely to undergo neoadjuvant chemotherapy. The average lipid profile of breast cancer patients in this cohort superimposes those of the sex, age and BMI-matched Portuguese population[499, 500.]

Results show that systemic LDL-C level above 117 mg/dL is a predictive factor of tumor T2 stage or higher, at diagnosis. These level is also positively associated with worse prognostic characteristics such as higher histological grade, higher proliferative rate[501] and more advanced clinical stage (II-III). Patients in the third tercile (LDL-C>144 mg/dl) are also more prone to have LVI and lymph node metastasis. This trend seems to be transversal to all immunohistochemical breast cancer subtypes, although we found a significant increase of HER2 positive cases in patients of the third tertile group.

Other published studies, also found higher TC levels in breast cancer patients, compared to healthy controls[403,406] as well as a trend of raised TC, LDL-C levels with higher tumor stage[403]. However, to our knowledge this is the first cohort of breast cancer patients in whom the correlation of lipid profile and tumor characteristics was done in a setting of pre-treatment and with all patients free of lipid lowering drugs. Despite we did not accessed variables that may also influence the lipid profile such as smoking habits, type of diet, residence area or socioeconomic status, the most important co-variables, BMI, age and

lipid lowering drugs were controlled. Furthermore, conversely to previous studies, this cohort includes patients in initial clinical stages and with the same histological type, in other to better control tumor variables.

Considering that proliferating cancer cells have an increased demanding of cholesterol and intermediates of cholesterol metabolism, the up-regulation of cholesterol biosynthesis and or an increased up take from the exterior are expected. Results support the hypothesis that high availability of systemic cholesterol promotes tumor growth and aggressiveness. However, observed high LDL-C levels may actually reflect a shift in cholesterol metabolism (in liver or tumor cells themselves) in patients with aggressive tumors, being a consequence of the tumor progression and not a causal factor.

In cancer cells, cholesterol synthesis has been shown to be increased, due to availability of precursors or to increased transcription of biosynthesis enzymes[502,503,504]. Hidroxi-3-methyl-glutaril-coA reductase 3 inhibition by statins decreases *in vitro* cell proliferation, attesting that cholesterol biosynthesis should be important to tumor growth[503]. Elevated cholesterol content is characteristic of breast tumors[505] and ACAT-1 inhibition, an enzyme involved in cholesteryl esterification decreases proliferation and invasion rate[411]. Although strong evidence that cancer cells increases intracellular cholesterol synthesis, this effect is not expected to produce hypercholesterolemia and justify the association.

On the other hand, high LDLR expression was shown in breast tumors compared to normal tissue[280] supporting that cancer cells are able to uptake LDL-C from the bloodstream. In *in vitro* experiments, HDL-C as well as exogenous triglycerides[276] were also demonstrated to be consumed by cancer cells through SR-BI[416,491,420] .

In order to support tumor needs, the exogenous cholesterol could be mobilized from body storage, through HDL-C or from diet, through hepatic metabolism and LDL-C. Accordingly, Zielinski et al[399] followed-up a group of patients with advanced breast tumors in remission and described a significant raise in plasma cholesterol and triglycerides in most of the patients of those who developed disease progression.

Our results does not exclude the possibility that LDL-C plasma levels are consequence of body mobilization, but we saw the same pattern of lipid profile in breast cancer patients and age and sex-matched non-cancer Portuguese population[499, 500] suggesting that the tumor, at this stage, is not changing LDL-C levels. Instead lipid profile of the study population may reflect the origin population.

Therefore, results strongly suggest that a tumor arising in an enriched-cholesterol environment has an advantage to progress (Figure 15), revealing that the host metabolic

features influence tumor behavior. However, the biological mechanisms underlying this association are totally unknown.

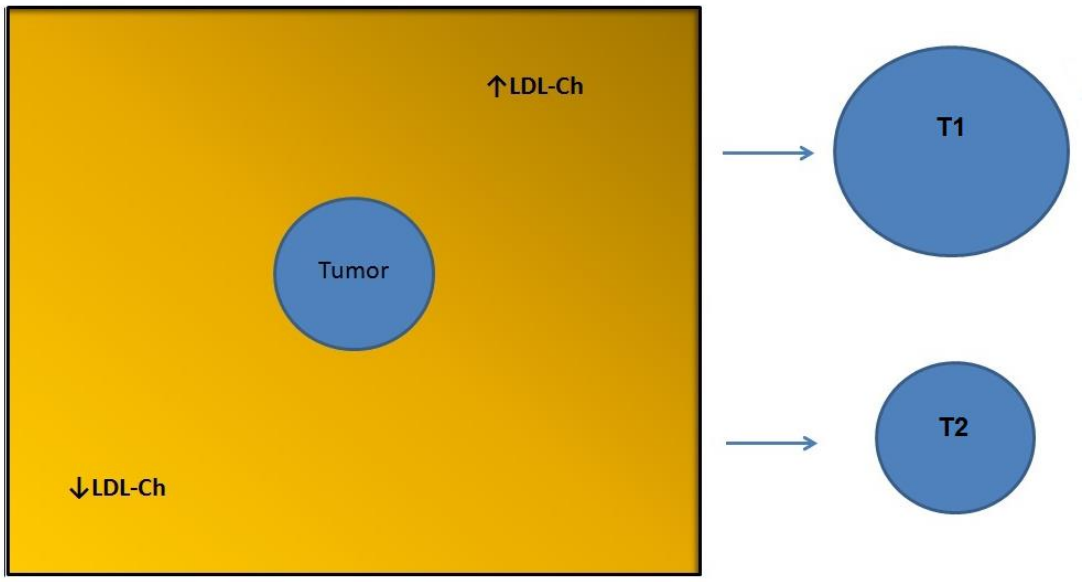


Figure 15: Phenotype of breast tumors exposed to high levels of cholesterol-Proposed Model.

We found that tumors of the LDL-C highest tertile are more commonly HER2 positive. Membrane cholesterol is specially localized in the lipid rafts domains. Such areas are enriched in transmembrane proteins that are key in signaling pathways associated with malignant progression: Fas receptor, TNF related apoptosis-inducing ligand, Akt, integrins, cadherins and growth factor receptors[256] including ERBB2. The last molecule is a tyrosine kinases receptor (and oncogene) localized in lipid rafts and it function is highly dependent on membrane fluidity[506,507]. Therefore cholesterol enrichment within the cell membrane may alter receptor-signaling. It is possible that ERBB2 receptor ligand-independent activation is potentiated by cholesterol-enriched environment, explaining selection of HER2 positive tumors.

Estrogen and progesterone are growth factors to ER/PR positive tumors and their synthesis requires cholesterol. Recent *In vivo* studies showed that oxysterol 27-hydroxycholesterol, also a derivate of cholesterol metabolism and ER-ligand, promotes ER positive breast cancer proliferation in mouse models[342]. In our study, no association of LDL-C and ER status was seen thus not supporting a fundamental mechanism ER-dependent.

The pro-inflammatory microenvironment induced by high-cholesterol levels, as seen in atherosclerosis, in which LDL-C is the most important causative factor[508,509], can also play

an effect on breast cancer initiation and progression. The use of statins before cancer diagnosis was associated to reduced cancer-related mortality[457]. This effect was supposed to be mainly due to the reduction of LDL-C, but statins anti-inflammatory mechanism cannot be ruled out.

Although the molecular mechanism by which systemic cholesterol exert an influence on the tumor is unknown, our results show that LDL-C level, at diagnosis is a predictive factor of tumor aggressiveness.

Conclusion

We found that LDL-C level at diagnosis is significantly associated with advanced and worse prognosis breast tumors and emerges as an important issue to investigate further.

Chapter 2

LDL-cholesterol signaling induces breast cancer proliferation and invasion[3]

Introduction

Cholesterol is an essential structural component of the cell membranes[256]. Proliferating cells, such as cancer cells, are believed to have increased requirements of cholesterol. According, cholesterol accumulation is a property of some malignancies[510,511] and the inhibition of cholesterol storage machinery, in breast cancer cell lines was associated with reduced proliferation[411].

To overcome their needs, tumor cells increase lipid biosynthesis[272], and may also uptake cholesterol from the bloodstream[276,288]. This ability of cancer cells to use exogenous lipids has been considered the link between high fat diets and dyslipidemia with cancer[276,411].

To date, most studies have seek to find a causal relation between cancer incidence and lipid levels[382,379,383,384,377,369,460], however less studies tried to explore a possible link in cancer aggressiveness or progression. Thus, for now, the importance of plasma cholesterol in cancer progression remains poorly understood and was the subject of the present study.

We asked whether exposure to a host LDL-C enriched systemic environment promotes breast cancer progression by activating key signaling pathways and modulating cell behavior. To test this hypothesis we used controlled experimental environments, employing well established *in vitro* and *in vivo* models.

[3] Results discussed in this chapter are published at Rodrigues dos Santos C, Domingues G, Matias I, Matos J, Fonseca I, de Almeida JM, Dias S **LDL-cholesterol signaling induces breast cancer proliferation and invasion** *Lipids Health Dis.* 2014 Jan 15;13(1):16. [Highly accessed paper, article is amongst the highest ever scored in this journal (ranked #49 of 312).]

Material and Methods[4]

Cell lines and reagents

The human breast cancer cell line HTB20 and the mouse breast cancer cell line 4T1 were purchased from the American Type Culture Collection. The human breast cancer cell lines HTB126, MDA MB 231 were kindly provided by Instituto Português de Oncologia do Porto.

The cell lines were cultured in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Invitrogen life Technologies). Fetal bovine serum lipoprotein free (FBSLF) was purchased from Sigma-Aldrich (Germany) and human plasma low density (LDL-C) and high density (HDL-C) lipoproteins were obtained from Calbiochem (Gibbstown, NJ, USA). Mitomycin C was from Sigma-Aldrich (Germany) and Trypsin from (Gibco Invitrogen, Carlsbad, CA, USA).

Cell proliferation assay

MDA MB 231, HTB 126, HTB 20 cells (1x 10^5/mL) were seeded into 24-well plate in 250µl DMEM,10%FBS. After overnight incubation, the medium was replaced by DMEM,1%FBSLF, for 24h. Then, the medium was aspirated and cells were incubated with medium containing HDL-C (100µg/mL) or LDL-C (100µg/mL), at 37ºC in 5%CO_2, for 24h or 48h. The number of living cells was determined by hemocytometer counts (at least 4 counts/well, in quadruplicates), after Trypan Blue test exclusion. The number of cells is expressed as fold change over the control.

Migration assay

MDA MB 231 cells were seeded on 24-well plate and grown to confluence in DMEM, 10%FBS. Upon reaching confluence, the medium was replaced by DMEM, 1%FBSLF, for 24h. Two-hundred-microliter tips were used to make a denuded area ("wound") in the center of the well. Each well was washed with PBS and treated with HDL-C (100µg/mL) or LDL-C (100µg/mL) for 24h. Mitomycin C (0,5µmol/L, from Sigma) was added to the medium to block cell proliferation. Serial photographs were taken at 0h, 12h and 24h, and cell migration distance was determined by subtracting the values obtained at 0h from 24h (at least 4 measurements/well, in quadruplicates). The migration distances are expressed as percentage of the wound closure.

[4] Assistance with RNA extraction and microarray analysis was obtained from Inês Matias. Western blott analysis was performed by Germana Domingues. Immunohistochemistry for Ki67 was performed by João Matos. The remainder experiments described in this chapter were performed by the candidate.

Adhesion assay

MDA MB 231 cells (1x 10^5/mL) were seeded into 24-well plate in 250µl DMEM, 10%FBS, overnight, and then replaced by DMEM, 1%FBSLF, for 24h. After 24h, cells were left untreated or exposed to LDL-C (100µg/mL), overnight. Then wells were washed with PBS, and cells removed with trypsin and reseeded into 24-well plates in 250µl DMEM,10% FBS, as defined earlier. After 4h, cells were washed with PBS and adherent counted. The cells in the supernatant were also counted. The results are shown as the number of adherent or supernatant cells/mL.

RNA extraction and microarray analysis

Total RNA was extracted by Trizol method from untreated or LDL-C treated breast cancer cells (MDA MB 231) and used to study changes in gene expression. The samples were hybridized on an Affymetrix GeneChips at Instituto Gulbenkian de Ciência core facility. The gene expression results were analyzed using Chipster 2.2.0 software. A cutoff of 1.5 fold above or below the house keeping gene expression levels was considered significant. IPA Ingenuity Systems (Ingenuity Systems, Mountain View, CA) was used to exploratory analysis of interactive networks and relevant biological interactions.

Protein extraction and western blotting analysis

Cells were lysed in 50 mM tris, 5 mM EDTA, 2% SDS, pH 6.8 buffer containing protease inhibitor cocktail. Lysates were diluted 1:1 in loading buffer (tris–glycerol, 2% SDS, 4% b-mercaptoethanol, 100 mM DTT) and 300µg protein was loaded on 10% tris–glycine gels. Proteins were transferred to 0.2lM nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences, Roosendaal, Netherlands) and subjected to standard immunoblotting with the antibodies: Akt (#4685), pAkt (#4060), ERK1/2 (#4695), pERK 1/2 (#4370),pJNK (#9251) and ß-actin (#A5441),all from Cell Signaling Technology Inc. Bands were detected with anti-species HRP conjugate. ImageJ software was used to quantify the density of the bands[512].

Statistical analysis

All results, unless otherwise indicated, are expressed as the mean±standard error of, at least, triplicates. Data were analyzed using unpaired two-tailed Student's t test. *P* values of <0.05 were considered statistically significant.

In vivo models

All animal experiments were performed after approval from Ethics Committee of the Instituto Gulbenkian de Ciência. Animals were housed and maintained in a barrier facility at Instituto Gulbenkian de Ciência. In each experiment, 4-6 week old female mice were

injected with breast cancer cells in the right axillary mammary fat pad. Than the test group was subjected to a high cholesterol diet (10%fat, 1,25%cholesterol, 0,5%Na cholate diet, Ssniff, Germany) and the control group fed the standard (normal) mouse diet, with no differences in energy up take values. Food and water were given *ad libitum*. Elevated cholesterol levels were confirmed by standard dosing methods at Clinical Pathology Laboratory of the IPOLFG and parallel groups were used to control the diet effect on lipid profile. In order to test different tumor types and different host backgrounds the following trials were performed:

BALB SCID/MDA MB 231 ($2x10^6$ cells, 4-6 week old female, n=8), 10 weeks;

BALB SCID/HTB 20 ($2x10^6$ cells, 4-6 week old female, n=4), 20 weeks;

NOD SCID/4T1 ($1x10^6$ cells, 4-6 week old female, n=4), 20 days;

BALB C/4T1 ($1x10^6$ cells, 4-6 week old female, n=5), 20 days.

The animals were sacrificed at different times following tumor inoculation, as referred above; mammary tumors, lungs and liver were excised. Tumors were split into two parts, one frozen in liquid nitrogen and stored at -80ºC and the other, and other organs, fixed in 10% neutral buffered formalin. Photos of the tumor were taken and the large diameter measured. Blood was collected, by cardiac puncture, and serum used to determine lipid profile (TC, LDL-C, HDL-C and triglycerides). Tumor sizes (large diameter, mm) are presented as fold change over the control group. Systemic metastases were searched macroscopically during organs collection and microscopically in lungs and liver.

Immunohistochemistry of tumors was made to Ki67 marker (M7240, Dako), and positive cells were counted a Dako Autostainer® using standard protocols, followed by counting positive cells in an automated cellular imaging system, at, Department of Pathology at IPOLFG.

An additional trial was done, using statins, in order to test the effect of the reduction of the systemic cholesterol on the tumor. Using as mice model NOD SCID /4T1, the test group was subjected to a high cholesterol diet (as described above) and treated with simvastatin (5mg/Kg in 200µL PBS, 3 days a week, (by gastric tube). Control groups were fed with standard (normal) mouse diet and high cholesterol diet and treated with placebo (200 µL PBS, 3 days a week, (by gastric tube). The mice were injected with tumor cells 4 weeks after starting treatment and treated during 4 weeks more. Animals were sacrificed as described before.

Statistical analysis

Values are given as the mean±standard error. Comparisons between control and test mouse samples were performed using the Student´s t test. The number of mice used for each experiment is indicated in the figure. *P* values of <0.05 were considered statistically significant.

Results

LDL-cholesterol stimulation induces breast cancer cell lines proliferation, migration and reduces cell adhesion

We examined the effect of LDL-C on breast cancer cells proliferation and found that the number of viable cells increased after LDL-C stimulation in all cell lines, reaching statistical significance in MDA MB 231 and HTB 20 at 48h (Figure 16 A). Further dissection of LDL-C induced phenotype was performed on MDA MB 231 cells. In detail, we also determined the effect of LDL-C in breast cancer cells migration in scratch wound healing assays (in the presence of Mitomycin C to inhibit cell proliferation) and found that LDL-C induced migration of MDA MB 231 cells, promoting *in vitro* wound closure. Importantly, HDL-C was used as control and did not promote cell migration (Figure 16 B and C).Thereafter we tested if cells incubated with LDL-C changed their adhesive behavior. As shown in Figure 16D and E, LDL-C pre-treated MDA MB 231 cells, lost their adhesion to the matrix compared to control (untreated) conditions.

Together, these data suggest that LDL-C exposure of breast cancer cells affects their adhesive properties, favoring cell migration and proliferation.

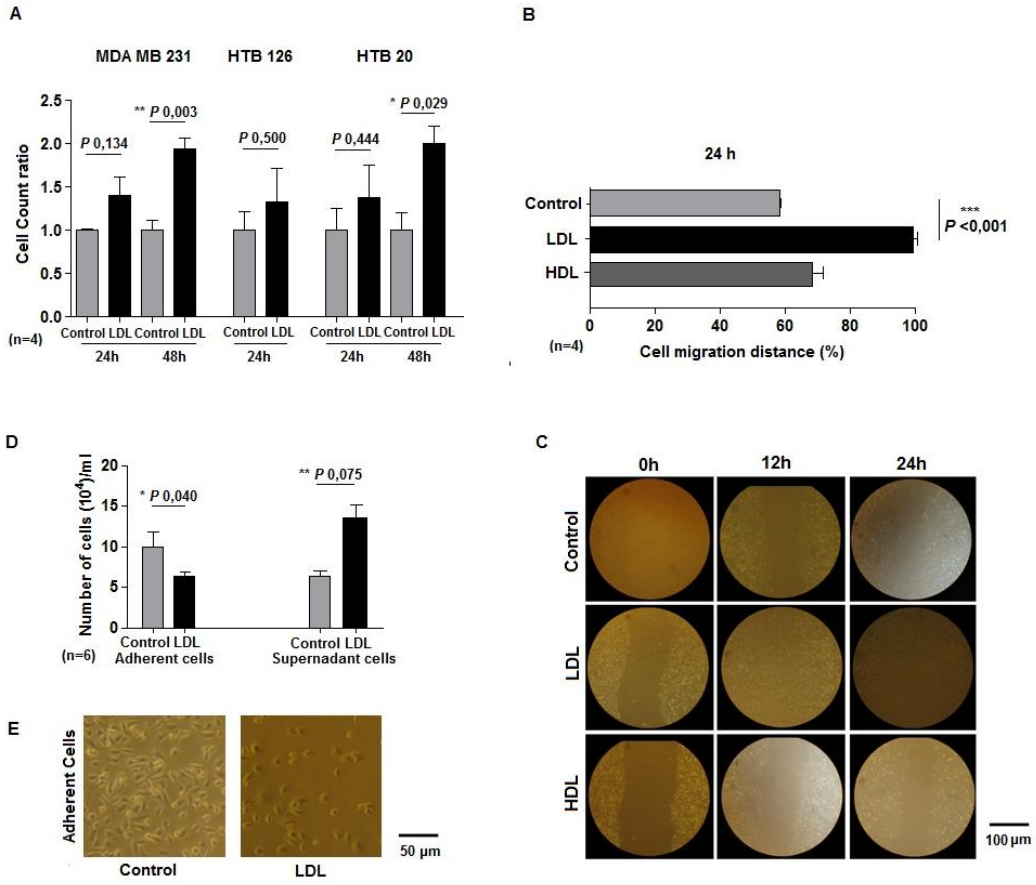


Figure 16: Proliferation, migration and loss of adhesion induced by LDL-C in breast cancer cell lines.

A. Number of cells (MDA MB 231, HTB 126, HTB 20) after 24h and 48h of LDL-C (100 µg/mg) exposure is consistently higher than control (non-treated) condition . **B** and **C**, Cells (MDA MB 231) were wounded and then cultured for 24h, with LDL-C (100 µg/mL), HDL-C (100 µg/mL) or control conditions. Cell migration into the wound was examined by phase-contrast microscopy and migration distance is indicated as the percentage of the wound closure at 24h. Representative photos are shown (original magnification 100x C). **D** and **E**, Number of cells (MDA MB 231) adherent and no adherent cells, at 4h after being removed and reseeded on its primary conditions (control and LDL-C100 µg/mL) shows that LDL-C treated cells lose matrix adhesion compared to control. Representative photos are shown (original magnification 200x E). *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-Cholesterol; HDL: HDL-Cholesterol.

LDL-cholesterol induced gene expression changes in breast cancer cell lines

Having shown that LDL-C induced phenotypic changes on breast cancer cells, next we sought to demonstrate a causal mechanistic link between these changes and the activation of signaling intermediates and pathways that could explain the altered properties. For this purpose we performed Affymetrix microarray analysis of untreated versus LDL-C treated breast cancer cells. As shown in Additional Table 3, we found an over expression (fold change ≥1,5) of 147 mapped genes and down regulation of 95 mapped

genes, at 48h. The great majority of these genes are related to cell survival and proliferation pathways. Among genes with altered expression there are the down regulation of adhesion molecules genes such as cadherin-related family member3 (-1,53 fold change), CD226 (-1,52 fold change), Claudin 7 (-1,52 fold change), Ocludin (-1,54 fold change) and integrinβ8 (-1,49 fold change). Exploratory analysis of the significant gene interactions, found an activation of Akt, ERK and JNK networks, all in the dependency of the ERBB2 pathway (Figure 17). By western blotting analysis we confirmed the increased phosphorylation of Akt and ERK, but not of JNK (Figure 18). Observed changes in gene expression and signaling pathways activation on LDL-C treated cells corroborate the LDL-C induced phenotype.

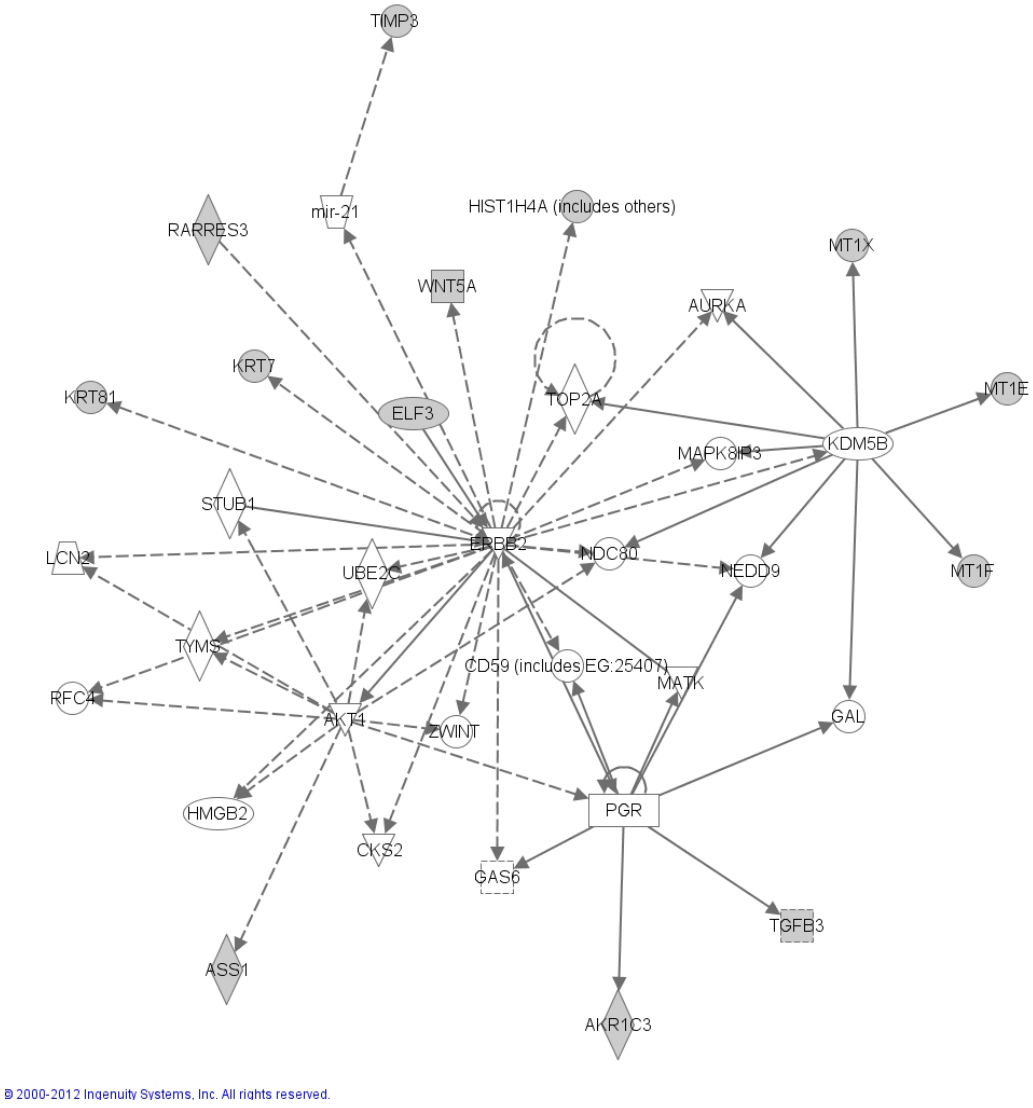


Figure 17: Activated cellular networks, at 48h, in LDL treated breast cancer cell line.

Gene expression analysis of breast cancer cells MDA MB231 exposed to LDL (100 µg/mL), for 48h shows up regulation of molecules involved in activation of ERK, Akt and ERBB2 pathways. Grey nodes are genes overexpressed, white nodes are predicted genes. Smooth lines represent direct interactions. Dashed lines represent indirect interactions.

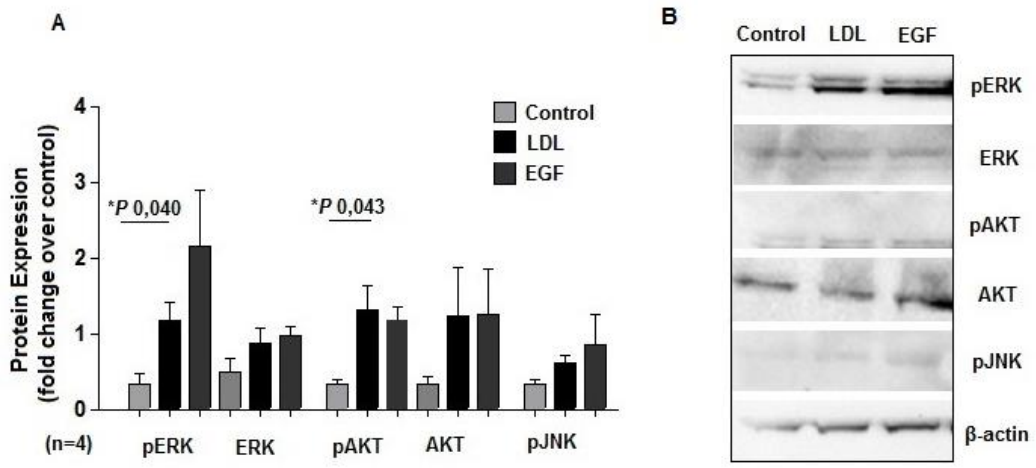


Figure 18: LDL-C induces ERK and Akt protein phosphorylation

A. Cells (MDA MB 231) exposed to LDL-C show higher expression of phosphorylated ERK and Akt, without significant increase in respective total protein. Phosphorylation of JNK is also higher, without reaching statistical significance. Epidermal growth factor (EGF) stimulation was used as positive control and induced, as expected, increase ERK, Akt and JNK phosphorylation. **B.** Representative photos of Western blot membranes are shown. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low density lipoprotein.

High LDL-cholesterol promotes breast cancer growth in animal models

HD fed mice showed high levels of TC, LDL-C and HDL-C (Figure 19 A) without statistically significant differences in triglycerides levels or animal weight (Figure 19 B). The values of the mouse lipid profile in each experiment are shown in Additional Table 4. These data validate this high fat diet model as a good model to specifically address the effects of elevated cholesterol levels.

HD promoted breast tumor growth in all models tested, with statistically significant differences in BALB SCID inoculated with MDA MB 231 and BALB C inoculated with 4T1 cells (Figure 19 C). Tumors of HD fed mice showed a proliferative ratio that was 20% higher than tumors from ND fed mice, assessed by immunohistochemistry (for Ki67 positive cells) (Figure 19 F and G).

Lung metastases were observed macroscopically and microscopically in the NOD SCID and BALB C/4T1 models. No significant differences were registered the first model, but BALB C/4T1 hypercholesterolemic mice showed a higher frequency of lung metastasis, at the end of the trial, compared to ND fed mice (Figure 19 E). No liver metastases were registered. Two mice of the longest BALB SCID/HTB 20 trial died, one of each group. No other mice deaths occurred.

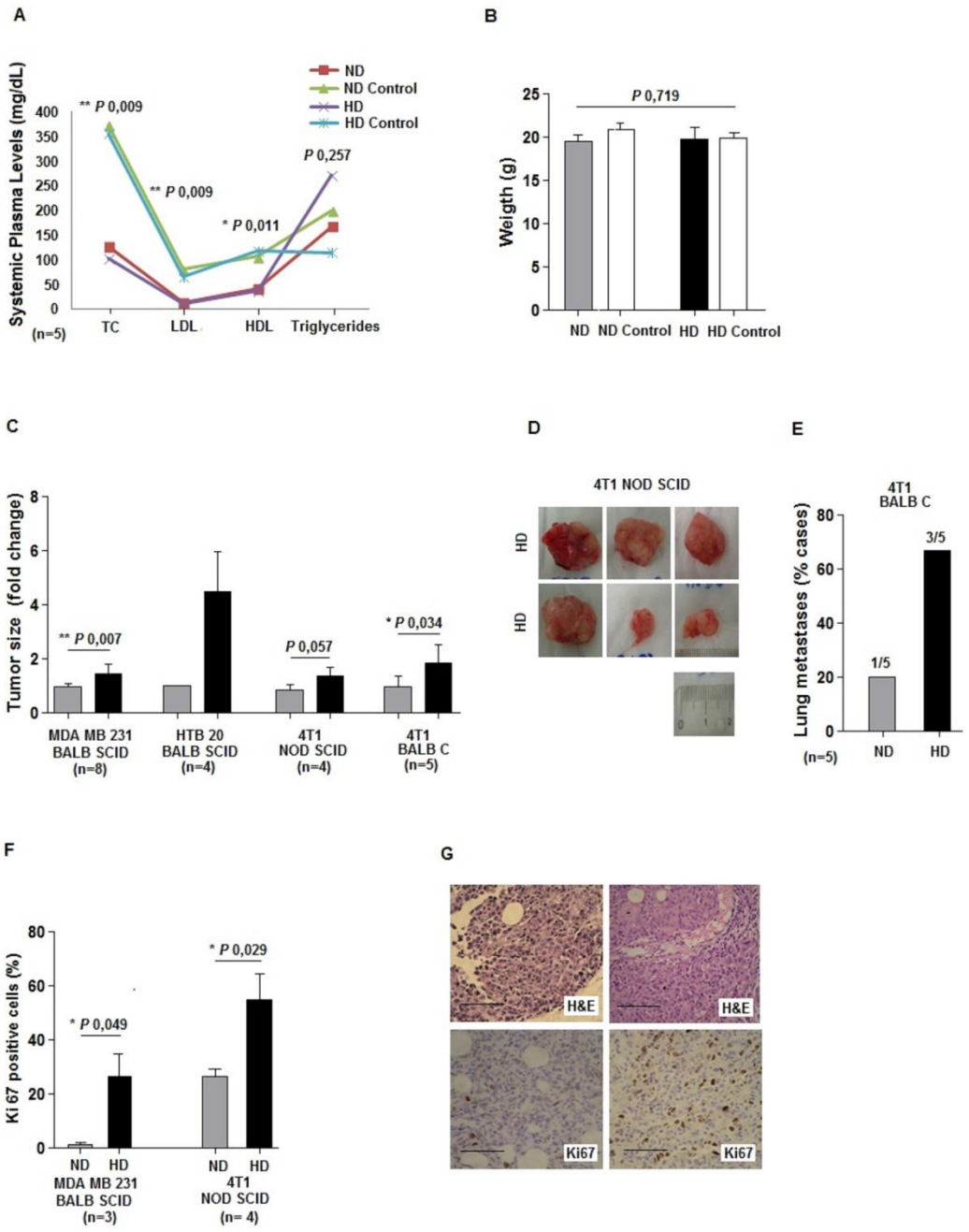


Figure 19: Hypercholesterolemic diet induces a breast cancer phenotype characterized by large and more proliferative tumors.

A and **B**. HD fed mice have raised levels of total cholesterol (TC), low density lipoprotein (LDL-C) and high density lipoprotein (HDL-C). There are no statistical significant differences in triglycerides level or animal weight, as exemplified to MDA MB 231/BALB SCID trial. (Results of the other experiments are in supplementary data.) Animal in the same diet but without tumor cells inoculation were used as control to lipid profile parameters. **C** and **D**. HD fed mice show large tumors when compared to ND fed mice. The mammary tumor large diameter was measured (as exemplified to 4T1 /NOD experiment B) and the differences are shown as the fold change over the ND fed mice. **E**. HD fed mice are more likely to have lung metastasis (100%) than ND fed mice (33%). **F** and **G**. HD fed mice have more proliferative tumors as confirmed by Ki67 immune staining. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low density lipoprotein.

Statins treatment does not reduced systemic LDL level in mice trial

The treatment with statins did not reduce cholesterol levels of HD fed mice (Figure 20 A). Tumor size of HD fed mice treated with statins were lower than HD fed mice not treated with statins , but without significant difference, in this trial (*P* 0,104) (Figure 20 B and C).

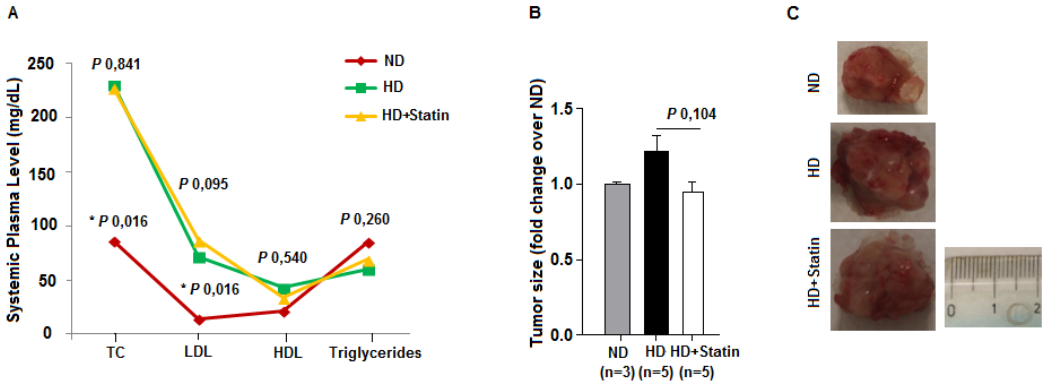


Figure 20: Tumor size of hypercholesterolemic diet fed mice treated with statins show no significant differences between hypercholesterolemic diet fed and control mice.

A. HD fed mice have raised levels of total cholesterol (TC) and low density lipoprotein (LDL:LDL-C) and no significant differences in high density lipoprotein (HDL:HDL-C) and triglycerides levels compared ND. Treatment with statins 5mg/dL, 8 weeks, does not change lipid profile in the HD fed mice. **B** and **C** NOD SICD/ 4T1 mice model fed with HD show no significant differences in tumor size compared to HD fed mice treated with statins 5mg/mL. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM.

Discussion

Obesity and dyslipidemia have long been linked with a possible increase in the likelihood of developing cancer. This possible causal relationship has gained momentum in the light of the observed “epidemic“ obesity and the recognized increased incidence of cancer in Western countries[40]. Moreover, the same trend in obesity, dyslipidemia and cancer incidence has been seen in Asian with the growing incorporation of Western lifestyles[4,210].

However, few studies have tried to find causal and mechanistic associations between increased lipid levels and- not cancer incidence- but cancer behavior. This was tested in the present study, using well established *in vitro* and *in vivo* models of hypercholesterolemia and breast cancer.

In breast cancer cell lines, representative of different tumor subtypes and stages, we found that LDL-C (but not HDL-C) exposure induces cell proliferation, migration and loss of adhesion, hallmarks of the process of epithelial to mesenchymal transition[183]. Others have also demonstrated that in some breast cancer cell lines, HDL-C induces

proliferation[513,514]. We saw a discrete effect of HDL-C in ER negative cells proliferation (data not shown, not significant), but no influence in migration or adhesion properties.

Previous studies, also showed that LDL-C induces proliferation[513,411] and migration[288] of ER negative, but not ER positive breast cancer cell lines suggesting that LDL effect is dependent on ER status. But, we used an ER, ERBB2 positive breast cancer cell line, HTB 20 (BT 474)[515], and LDL-C induced similar phenotype changes.

Gene and protein expression analysis of breast cancer cells stimulated with LDL-C revealed that the proliferative effect induced by LDL-C may be dependent on Akt and ERK pathways activation. Gene expression analysis also suggested decreased expression of adhesion molecules such as cadherin-related family member 3, CD226, Claudin 7 and Ocludin, upon breast cancer cells exposure to LDL-C. These findings could explain the loss of adhesion in the functional tests, when cells were exposed to LDL-C.

Our own data shows an association between high plasma LDL-C level and ERBB2 positive breast cancers[516]. Also the exploratory analysis of gene expression microarrays suggested an upstream activation of an epidermal growth factor receptor (EGFR). Human epidermal growth factor receptor-2 (Her-2/neu or ERBB2) is a membrane tyrosine kinase and oncogene that is overexpressed and gene amplified in about 20% of breast cancers[517]. Independent investigations have demonstrated that cell cholesterol depletion using methyl-N-cyclodextrin, reduces fluidity of the membrane and enhances phosphorylation and consequent activation of EGFR downstream cascade[251,518,507,519]. Orr et al[507], specifically demonstrated this effect in ERBB2 receptor. Since EGFR could be activated in a ligand-independent manner[519], we propose as a mechanism that cholesterol mobilization across the cell membrane may be the responsible for changes in membrane equilibrium/ disorganization leading to ERBB2 activation, rather than the absolute cholesterol content itself. As mentioned earlier, this possibility remains to be fully exploited in future studies.

To systematically test the *in vitro* results, hypercholesterolemia, controlling obesity, was induced in mice of different background (including NOD SCID mice, which are non-obese mice) with different cells lines (to mimic different tumor subtypes) and data consistently showed greater tumor growth in the high LDL-C groups. This is in accordance with the results reported by Llaverias et al, in breast and prostate genetic mice models[44,520]. Our xenograft models have the advantage of being more representative of breast cancer heterogeneity.

Tumors grown in hypercholesterolemic mice have higher proliferative ratios, measured by Ki67 immunostaining, which is considered a worse prognostic marker[501]. Higher frequency of lung metastasis was also observed in HD fed mice inoculated with the 4T1 xenogeneic breast cancer cell line. In models using human breast cancer cell lines we were not able to detect systemic metastasis, either macro or microscopically, perhaps because those cells do not induce metastasis in mice (reports are very scarce) or because the length of our experiments was not sufficient.

Thereafter, we tried to reverse the hypercholesterolemic-induced phenotype by treating mice with lipid lowering drugs. However we did not achieve systemic LDL-C levels reduction with statins, in our model. This is a limitation of our work but such difficulty was also found by others[521]. Rats and mice commonly used in experimental cancer studies are generally unresponsive to the hypocholesterolemic effects of statins[521].

In humans, the effect of statins in cancer prevention and treatment remains controversial. Two large meta-analyses from 2006 described a neutral effect of satins in cancer[445,446]. A very recent cohort study crossing statins prescription/ pharmacy dispense and cancer related-mortality in Denmark National databases[457], proved that statins use before diagnosis of cancer is associated with reduced cancer-related mortality. Although some limitations were found in the study and pointed by the authors and others[458], such as lack of follow up and co-morbidities information, the association seems to be plausible, in the study population.

The real effect of statins in cancer cells is not well known. Statins decrease intracellular cholesterol synthesis by targeting HMGR. Theorically they lower the products of mevalonate pathway involved in cell proliferation and by this mechanism prevent tumor progression. While this effect was demonstrated *in vitro*, there is poor evidence of direct action of statins in tumor cells[522] when given orally, *in vivo*, and the effect is supposed to be more dependent of cholesterol levels. Therefore, much remains to be learned and developed with regards to lipid control in breast cancer setting.

Taken together, our findings show that breast tumors exposed to a LDL-C-rich host macroenvironment may be in survival advantage, which will ultimately result in a more aggressive cancer phenotype (Figure 21). Our results are supported by functional studies in cell lines and animal models of breast cancer and are in strong accordance with clinical data. The study exposes the importance of controlling systemic cholesterol levels in breast cancer prevention and treatment revealing LDL-C as a biomarker of tumor aggressiveness.

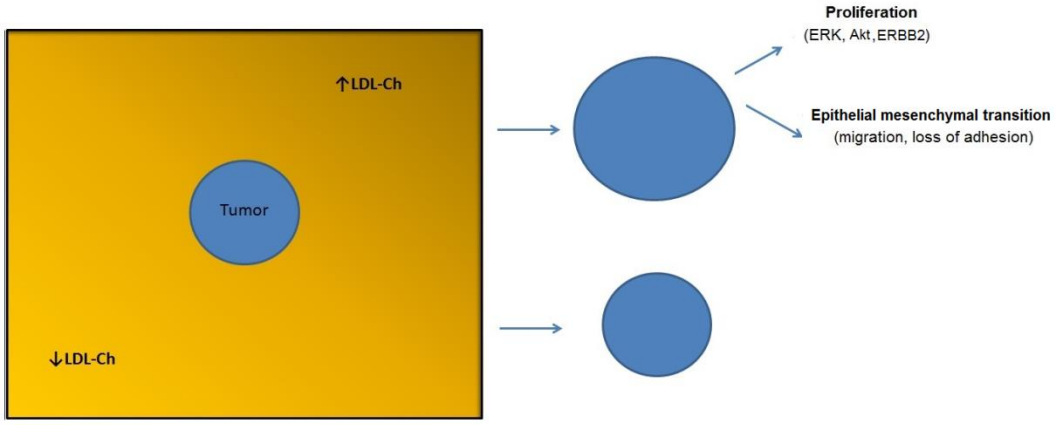


Figure 21: Phenotype of breast tumors exposed to high levels of cholesterol-Proposed Model.

Breast tumors exposed to high levels of LDL-cholesterol are larger and show increased proliferation and migration while losing epithelial adhesion properties.

Chapter 3

ABCA1 silencing reduces breast cancer aggressive phenotype induced by LDL-cholesterol[5]

Introduction

Altered lipid metabolism has increasingly been recognized as a common property of malignant cells. It is modulated by oncogenic signaling pathways and is important for initiation and progression of cancer, since cellular proliferation is dependent on sustained availability of lipids[136, 272]. Among lipid metabolism alterations, *de novo* biosynthesis has been posited to play a major role in cancer[272] but, there is emerging evidence that exogenous lipids may also be incorporated by tumor cells, contributing to malignancy[276, 288]. This metabolic feature has been considered an explanation for the association of some cancers and high fat diets.

In fact, we previously revealed that high level of systemic LDL-C, a common consequence of high fat diets, is positively associated with large and high grade breast tumors, as well as with reduced disease-free survival[516]. Moreover, cholesterol-enriched macroenvironment selects subsets of cells able to up take the exogenous cholesterol and thereafter promote cell proliferation, migration and EMT[523].

Cholesterol is a pivotal cell component, especially to membrane microdomains- lipid rafts[256] and essential to cellular functions as signal transduction, intracellular trafficking, polarity and cell migration[256].

However, despite the relevance of cholesterol in regulating such fundamental aspects of cell biology, its excessive intracellular accumulation is deleterious and can promote dysfunction of cell membrane proteins and domains[284], induction of caspase-mediated cell death[285, 286], organelle disruption and oxidative damage[287]. Cells that rely on endogenous biosynthesis do not accumulate excess of cholesterol because of homeostatic regulation of the cholesterol biosynthesis pathway. Cells dependent on exogenous cholesterol are more exposed to the environment and, besides repressing endogenous synthesis; they need other mechanisms to prevent cholesterol accumulation.

One mechanism of cellular protection is the esterification mediated by ACAT-1. It was already described that some breast cancers show high content of lipids and up regulation of ACAT-1[288,411]. Nevertheless, if cholesterol influx exceeds the capacity of ACAT-1, free

[5] Results discussed in this chapter are included in a manuscript in preparation.

cholesterol accumulates in the endoplasmatic reticulum membrane where the enzyme localizes, compromising ACAT-1 activity[524].

Another protective mechanism is cellular efflux of cholesterol, mainly through ABC transporters[525]. In peripheral tissues, ABCA1 is the main membrane transporter, responsible for the efflux of free cholesterol and phospholipids[525]. If this mechanism is used by breast cancer cells and wether it is relevante for tumor progression is not well known.

Systemic cholesterol induces an aggressive phenotype in breast cancer cells by selecting clones prone to use the available cholesterol. We hypothesized that the perturbation of cholesterol flux equilibrium, namely ABCA1 efflux mechanism, might disrupt the hypercholesterolemic induced aggressive phenotype.

Material and Methods[6]

Cell lines and reagents

The murine breast cancer cell line 4T1 was purchased from the American Type Culture Collection and the human breast cancer cell lines HTB20, MDA MB 231 were kindly provided by Instituto Português de Oncologia do Porto.

Cells lines were cultured in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Invitrogen life Technologies). Lipoprotein deficient serum from fetal calf (LPDS), Glyburide, Mitomycin C and DMSO were purchased from Sigma-Aldrich (Germany), human plasma low density (LDL-C) and high density (HDL-C) lipoproteins were obtained from Calbiochem (Gibbstown, NJ, USA), and Trypsin from (Gibco Invitrogen, Carlsbad, CA, USA).

The antibodies used were: Akt (#4685), pAkt (#4060), ERK1/2 (#4695), pERK 1/2 (#4370), pJNK (all from Cell Signaling Technology Inc; ABCA1 (ab18180, Abcam); LDLR (ab30532, Abcam) Ki67 (M7240, Dako) and ß-actin (#A5441, Sigma-Aldrich); Alexa Fluor® 488 (A-21202) and 594 (A-21203) from Invitrogen life Technologies.

[6]Assistance with RNA interference experiments was obtained from Jacinta Serpa and Ana Costa. Western blott analysis was performed by Germana Domingues. Immunohistochemistry was performed by João Matos and reviewed by Isabel Fonseca. The remainder experiments described in this chapter were performed by the candidate.

Human samples analysis

A total of 44 patients with breast invasive carcinoma, NOS[493], treated at Multidisciplinary Breast Unit of the IPOLFG during the year of 2011 and enrolled in a published cohort[516] were studied concerning ABCA1 expression in primary breast tumor. Patient samples were selected from 14 consecutive patients of each LDL-C tertile group of the studied population (each tercile group represents groups of patients with increasing systemic LDL-C levels).

ABCA1 (1:100; ab18180 from Abcam) staining was performed in breast cancer samples, using testis tissue as positive control. Measurements of ABCA1 expression was based on topographic score (0 to +3) of antibody staining tissue distribution accessed under microscopy by two independent observers. Technicians and pathologists were blinded to the lipid profile status of the study participants. This study was approved by the Ethics Committee of IPOLFG.

Cell proliferation assay

MDA MB 231 and HTB 20 cells ($1x10^5$/mL) were seeded into 24-well plate in 250µl DMEM,10%FBS. After overnight incubation, the medium was replaced by DMEM,1%LPDS, for 24h or 48h. Then, the medium was aspirated and cells were incubated in different conditions: Control (DMEM+1%LPDS), LDL-C (DMEM+1%LPDS+LDL-C100µg/mL), DMSO (DMEM+1%LPDS+0,01%DMSO), GLY (DMEM+1%LPDS+Glyburide 200µM/mL), LDL-C+GLY(DMEM+1%LPDS+ LDL-C 100 µg/mL+ Glyburide 200µM/mL), at 37ºC in 5%CO_2, for 24h or 48h. The number of living cells was determined by hemocytometer counts (at least 4 counts/well, in quadruplicates), after Trypan Blue test exclusion. The number of cells is expressed as fold change over the control.

Apoptosis and cell death

Apoptosis of MDA MB 231 cells were detected after treatment with glyburide, or control for 48h. Phosphatidylserine exposure on the surface of apoptotic cells was identified by flow cytometry (BD FACS Calibur™, BD Biosciences) after staining with Annexin V-FITC (Biolegend) and propidium iodide (PI,50μg/ml). In parallel the number of death cells was evaluated in the cell proliferation assays of MDA MB 231 and HTB 20 cells, by using Trypan Blue test.

Migration assay

MDA MB 231 cells were seeded on 24-well plate and grown to confluence in DMEM,10%FBS. Upon reaching confluence, the medium was replaced by DMEM, 1%, for

24h. Pipette tips (200 µl) were used to make a denuded area (“wound”) in the center of the well. Each well was washed with PBS and treated with the same conditions as in the cell proliferation assay. Mitomycin C (0,5µmol/L, from Sigma) was added to the medium to block cell proliferation. Serial photographs were taken at 0h, 12h and 24h, and cell migration distance was determined by subtracting the values obtained at 0h from 24h (at least 4 measurements/well, in quadruplicates). The migration distances are expressed as percentage of the wound closure.

Cholesterol measurements

The measurement of intracellular and culture medium cholesterol content was made by using the Amplex® Red Cholesterol assay Kit (Invitrogen) on thin layer chromatography[526].

Quantification of mRNA levels

RNA was extracted by Trizol (Sigma) method from untreated or treated breast cancer cells. cDNA was sinthetized with Superscript II (Invitrogen, Carlsbad, CA) by using random-sequence hexamers primers (Roche Applied Science, Indianapolis, IN). Real-time PCR was performed with Power SYBR Green PCR Master Mix in 7900HT Fast Real-Time PCR System (both from Applied Biosystems, Foster City, CA). Amplification of 18S RNA was used for sample normalization.

RT-PCR data were analyzed by DataAssist software (Applied Biosystems Foster City, CA) using RNA 18S as endogenous control. The final results are expressed as fold relative differences in gene expression between the studied samples and the control sample (calibrator). The following are the primer sequences used; 18S RNA forward GCCCTATCAACTTTCGATGGT reverse CCGGAATCGAACCCTGATT; ABCA1 RNA forward AACGCCCTCACCAAAGACCCT reverse AGGGCGTGTCTGGGATTGGG; HMGR RNA forward CCAAACCCCGTAACCCAAAG reverse AGCGACTATGAGCGTGAACAA; LDLR RNA forward GCTTGTCTGTCACCTGCAAA reverse AACTGCCGAGAGATGCACTT.

Western blotting analysis

Treated and untreated cells were collected at 48 hours and lysed with RIPA buffer (20mM Tris pH 7.5, 150mM NaCl, 5mM KCl, 5mM MgCl, 1% Triton X-100, protease inhibitor cocktail and 1mM sodium orthovanadate). Equal amounts of proteins (100µg/lane) were subjected to 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-Protean TGX precast gel, BioRad, US). Proteins were transferred onto nitrocellulose membrane (Hybond-C Extra, GE Healthcare Life Sciences, Roosendaal, Netherlands) and subjected

to standard immunoblotting with primary antibodies against to ABCA1 (1:1000), Akt (1:1000), pAkt (1:1000), ERK1/2 (1:1000),pERK 1/2 (1:2000), pJNK (1:1000) and ß-actin (1:5000).

Small interfering RNA

Cells (MDA MB 231) were seeded into 24 wells plate ($2x10^4$ / well) and transfected with ON-TARGET plus non-targeting Pool (D-001810-10-05, Dharmacon; therefore named control, scramble) and with ON-TARGET plus SMART pool, Human ABCA1 (L-004128-00-0010, Dharmacon). After transfection, cells were incubated for 48h, at 37ºC in 5% CO2 atmosphere in control and LDL-C conditions.

Immunofluorescence

Cells treated and untreated, cultured over a glass slide and were tested to the ABCA1 expression by Immunofluorescence. Primary antibody was incubated at room temperature for 1h; secondary antibody was incubated at room temperature for 2h. Slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

In vivo models

All animal experiments were performed after approval from Ethics Committee of the Instituto Gulbenkian de Ciência. Animals were housed and maintained in a barrier facility at Instituto Gulbenkian de Ciência. Mice model were established by injecting 4T1 cells ($1x10^5$) ^{in} the right axillary mammary fat pad of 4-6 week old female mice, Balb/c. Then, animals were subjected to a high cholesterol diet (10%fat, 1,25%cholesterol, 0,5%Na cholate diet, Ssniff, Germany) and the other group fed the standard (normal) mouse diet, with no differences in energy up take values. Food and water were given *ad libitum*. Elevated cholesterol levels were confirmed by standard dosing methods (at Pathology Laboratory of IPOLFG) and parallel groups were used to control the diet effect on lipid profile. Five days after tumor inoculation, Glyburide (10mg/kg) was administered through intraperitoneal injection in alternate days regimen. Glyburide was prepared with glyburide 0,5mg/ml, DMSO 0,5%, Ethanol10%, PBS 39,5%, PEG 400 50%. The vehicle solution was used as placebo in the control group.

The animals were sacrificed 20 days after tumor inoculation. Mammary tumor, lungs and liver were excised. Tumors were split into two parts. One part frozen in liquid nitrogen and stored at -80ºC.The other part, lung and liver were fixed in 10% neutral buffered formalin. Photos of the tumor were taken and the volume measured (side x side x side, mm^2). Blood was collected, by cardiac puncture, and serum used to determine lipid profile (TC,

LDL-C, HDL-C and triglycerides) and glycemia. Lung metastases were assessed by microscopic evaluation of serial sections.

Statistical analysis of experimental data

Values are given as the mean±standard error. Comparisons between control and test samples were performed using unpaired two-tailed Student's t test. P values of <0.05 were considered statistically significant. The number of mice/experiments used for each trial is indicated in the figure. P values of <0.05 were considered statistically significant.

Statistical analysis was performed using Graph Pad Prism version 5.00 for Windows, Graph Pad Software, San Diego California USA.

Results

Exogenous LDL-cholesterol induces ABCA1 expression

We started by observing a significantly elevation of ABCA1 mRNA expression in LDL-cholesterol treated cells compared to non-treated cells (Microarray analysis, data not shown). ABCA1 gene expression differences were confirmed by RQ-PCR, revealing that ABCA1 gene expression in LDL-C treated cells is 4,5 fold higher than control cells, *P* 0,004 (Figure 22 A).

Differential expression of ABCA1 protein was demonstrated by Western blot analysis of LDL-C treated MDA MB 231 cells (Figure 22 B) confirmed by immunofluorescence to MDA MB 231 (Figure 22C) and also observed on HTB 20 (Figure 22 D) breast cancer cell lines, representative of different breast cancer subtypes.

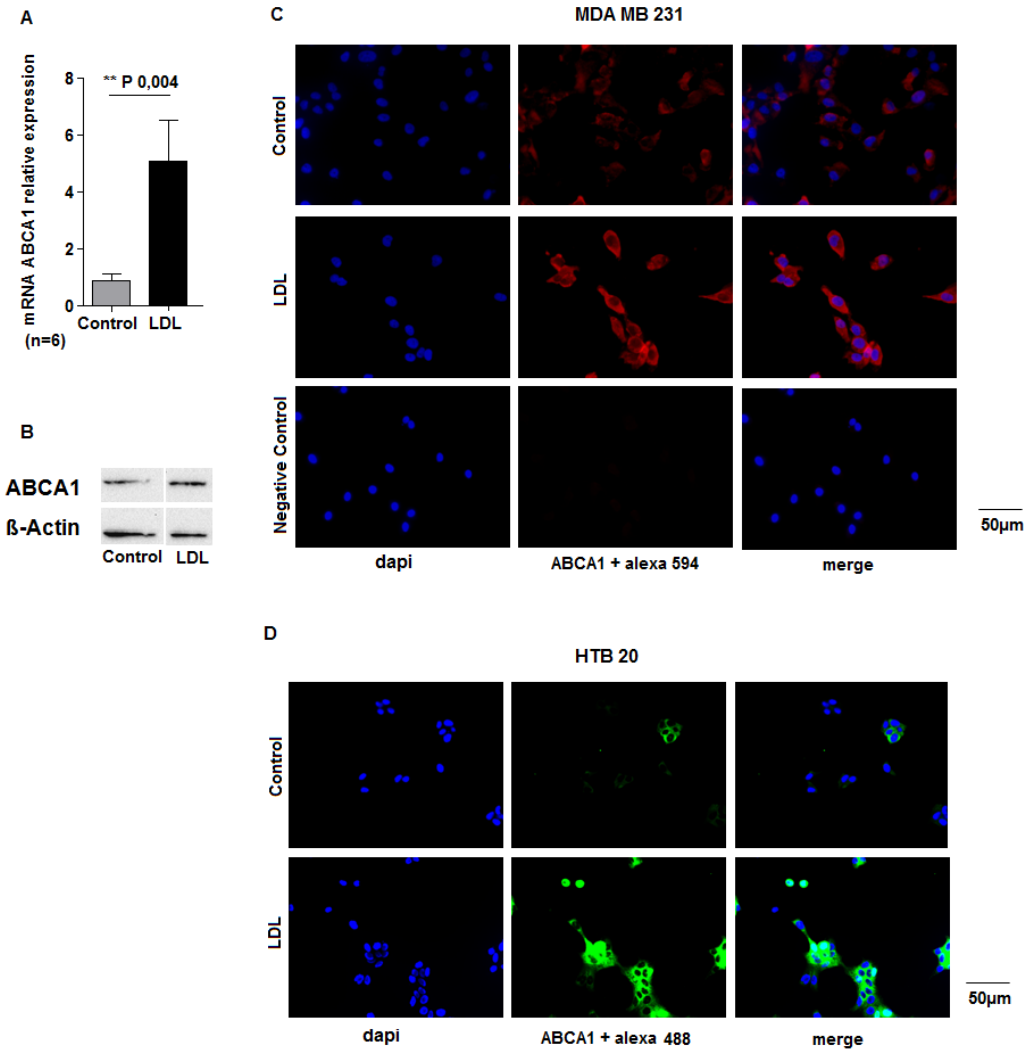


Figure 22: Exogenous LDL-cholesterol induces ABCA1 expression.

A. Total amount of mRNA of ABCA1 was measured by RT-PCR using mRNA derived from MDA MB 231 cells treated with LDL (100 ug/dl) and from control non treated cells. ABCA1 gene is overexpressed in LDL treated cells, at 48h (*P* 0,004). **B, C** and **D**. ABCA1 protein expression was detected by western blot and by immunofluorescence in MDA MB 231 treated and non-treated cells and by immunofluorescence in HTB 20 treated and non-treated cells. Representative photos suggest a correspondent overexpression of ABCA1 in LDL-C treated cells Immunofluorescence photos (X40). *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein.

Systemic level of LDL-C correlates with ABCA1 expression

In order to study ABCA1 expression in human breast cancer tumors, we analized ABCA1 expression in human samples of breast tumors of patients with increasing levels of systemic LDL-C levels and found that ABCA1 protein is present in almost all studied breast tumors, but expression is higher in tumors of patients with elevated LDL-C levels (Figure 23)

Of interest, exploratory correlations of ABCA1 with tumor immunohistochemical subtypes did not show significant differences. However, is important to note that the number of sample was small and not designed to measure differences.

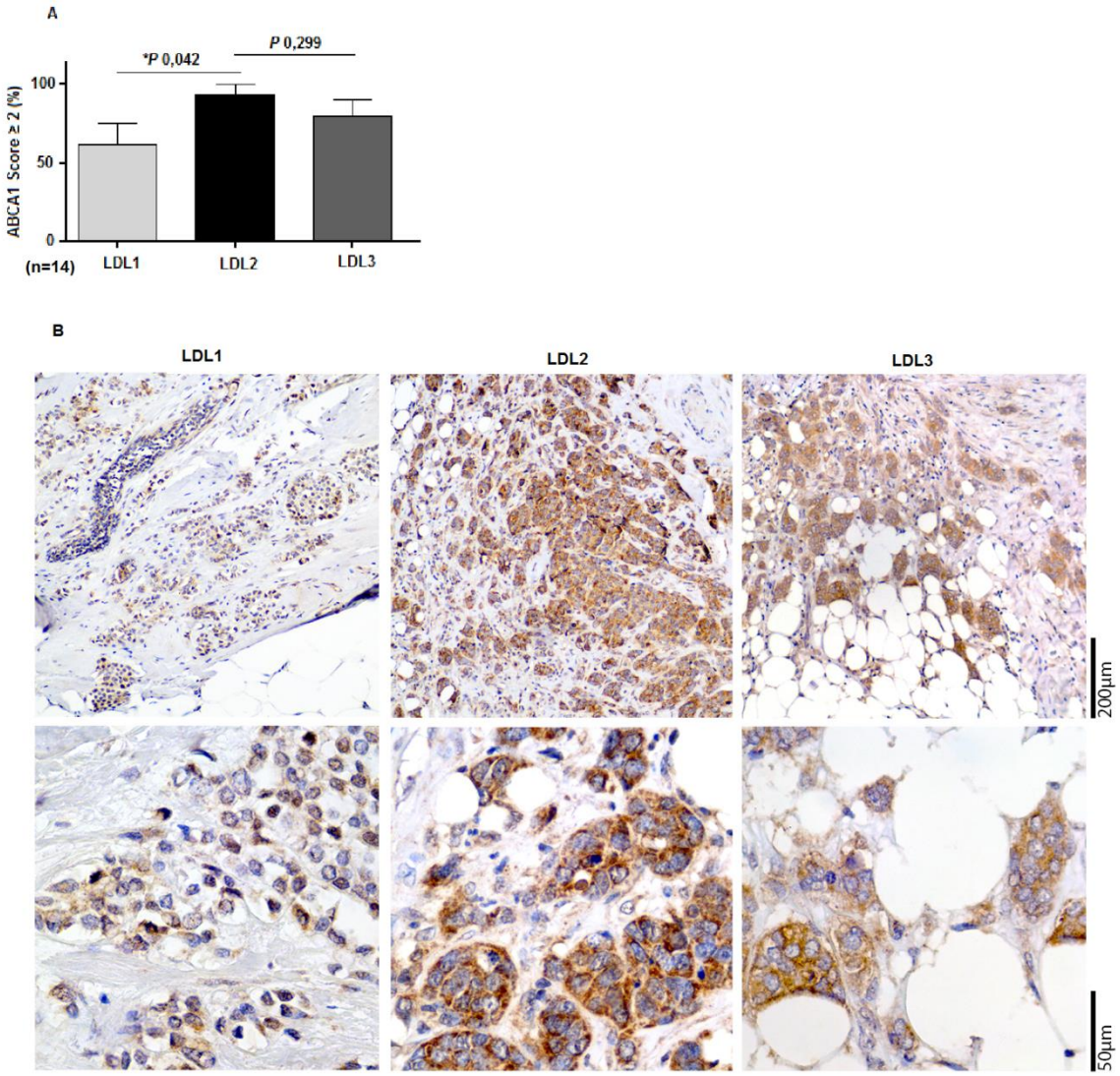


Figure 23: ABCA1 expression in human breast tumors

ABCA1 expression is higher in primary tumors of patients patients with elevated levels of systemic LDL-C (LDL2 and LDL3 in which LDL-C≥117mg/dl). Representative photos of each group are shown. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein.

ABCA1 inhibition reduces LDL-cholesterol-induced proliferation and migration and increases apoptosis of breast cancer cells

Having shown that exogenous LDL-C induces proliferation and migration of breast cancer cell lines[523] which courses with overexpression of ABCA1 (Figure 22), we sought to demonstrate the effect of ABCA1 inhibition on this phenotype. For this purpose we used glyburide, which is a selective inhibitor of ABC transporters[527,528,529].

Inhibition of ABCA1 reduces cell proliferation (Figure 24 A and B) and cell migration (Figure 24 C and D). DMSO was used as vehicle solution to glyburide and because of that control DMSO condition is shown. Neither vehicle nor the inhibitor drug produces significant effect on cell death (Figure 25 A and B). However ABCA1 inhibition significantly increases apoptosis in LDL-C exposed cells treated with glyburide, when compared to non-treated cells (Figure 25 C).

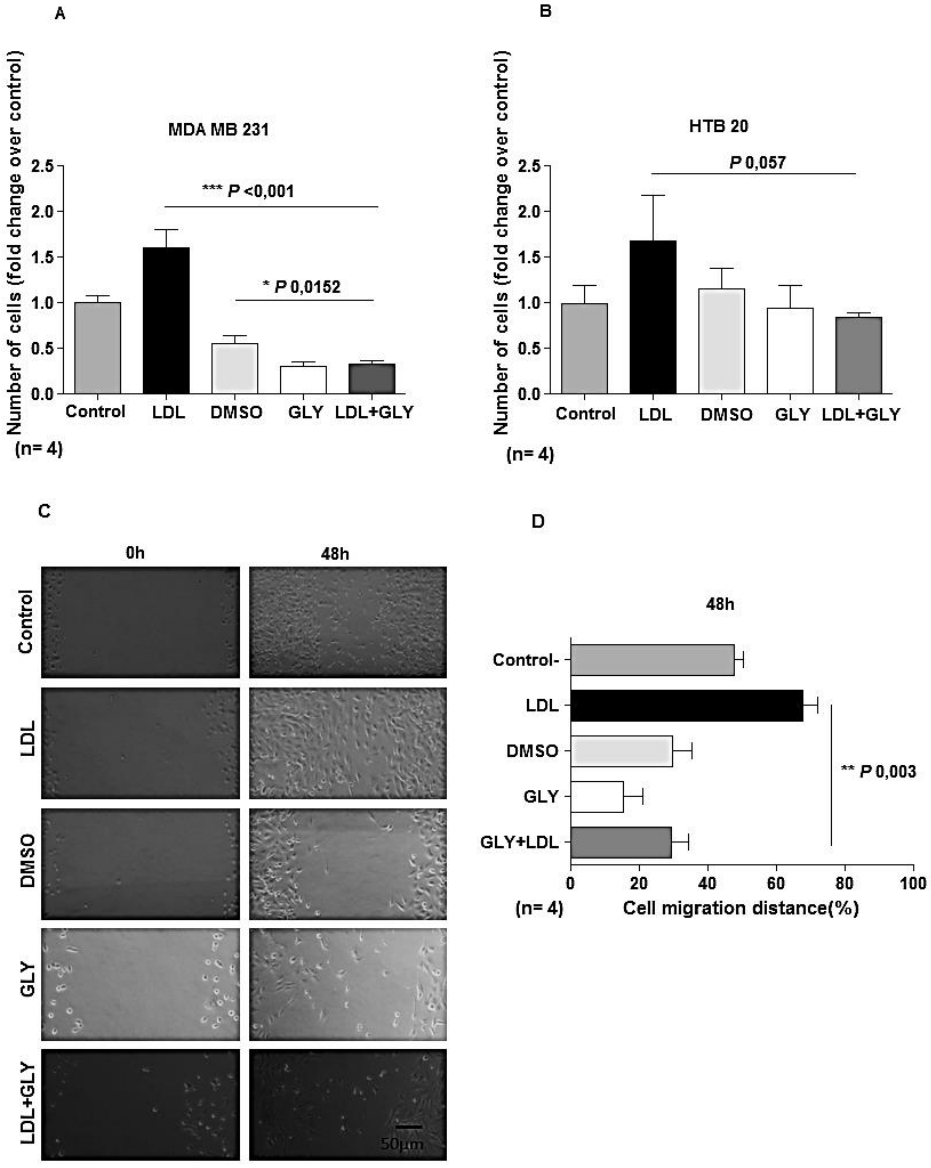


Figure 24: ABCA1 inhibition reduces LDL-induced proliferation and migration of breast cancer cell lines.

A and **B**. LDL-C exposure induces cell proliferation. Glyburide treatment reduces the number of cells when exposed to LDL-C, with significant difference in MDA MB cells at 48h (*P* 0,0182) and an evident trend in HTA 20 cells, at the same time point. **C** and **D.** Cell migration into the wound was examined by phase-contrast microscopy and migration distance is indicated as the percentage of the wound closure at 48h. . LDL-C induces cell migration and treatment of LDL-C- exposed cells with glyburide significantly decreases (*P* 0,003) the ability of MDA MB cells to migrate. Representative photos of wound healling assays are shown (original magnification 100xC). *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide.

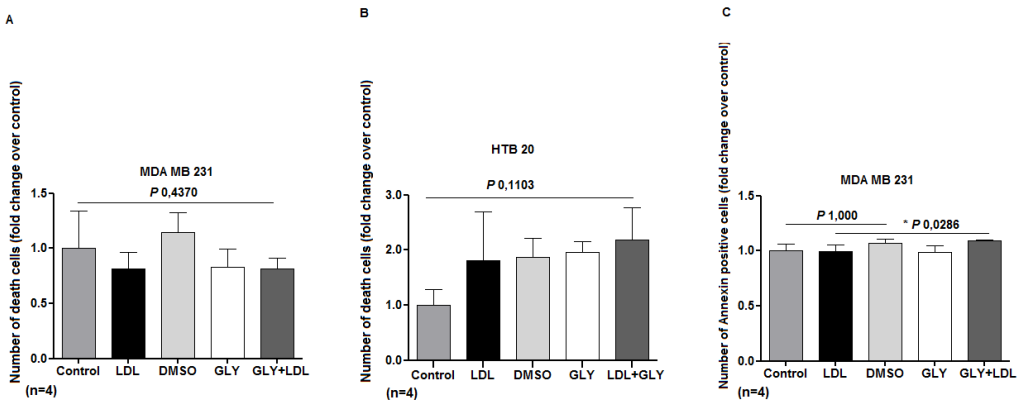


Figure 25: Apoptosis and cell death.

A and **B**. Breast cancer cells (MDA MB 231 and HTB 20) were cultured in control (DMEM+1%LPDS) or in LDL-C enriched medium (DMEM+1%LPDS+LDL-C100µg/mL) and then treated with glyburide (GLY (DMEM+1%LPDS+Glyburide 200µM/mL), LDL-C+GLY(DMEM+1%LPDS+ LDL-C 100 µg/mL+ Glyburide 200µM/mL), at 37ºC in 5% CO_2, for 48h. DMSO was used as vehicle to glyburide and so, control condition with DMSO (DMEM+1%LPDS+0,01%DMSO)is also shown. The number of inviable cells determined by hemocytometer counts (at least 4 counts/well, in quadruplicates) by Trypan Blue test, shows that treatment with glyburide does not produce differences in cell death, at 48h. **C**. Apoptosis was measured in MDA MB 231 cells, cultured in similar conditions after staining with Annexin-PI. Annexin positive cells were counted by Fluorescence –activated cell sorting. The number of cells is expressed as fold change over the control. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide.

To specifically demonstrate the ABCA1 down regulation and because ABCA1 protein expression and mRNA levels are discordant in different cells and tissues[530], we investigated the effect of glyburide in ABCA1 mRNA expression by RT-PCR, as well as protein expression by immunofluorescence and Western blot analysis. Results showed that glyburide produces an important down expression of ABCA1 transcription and visibly decreases the expression ABCA1 the protein levels (Figure 26).

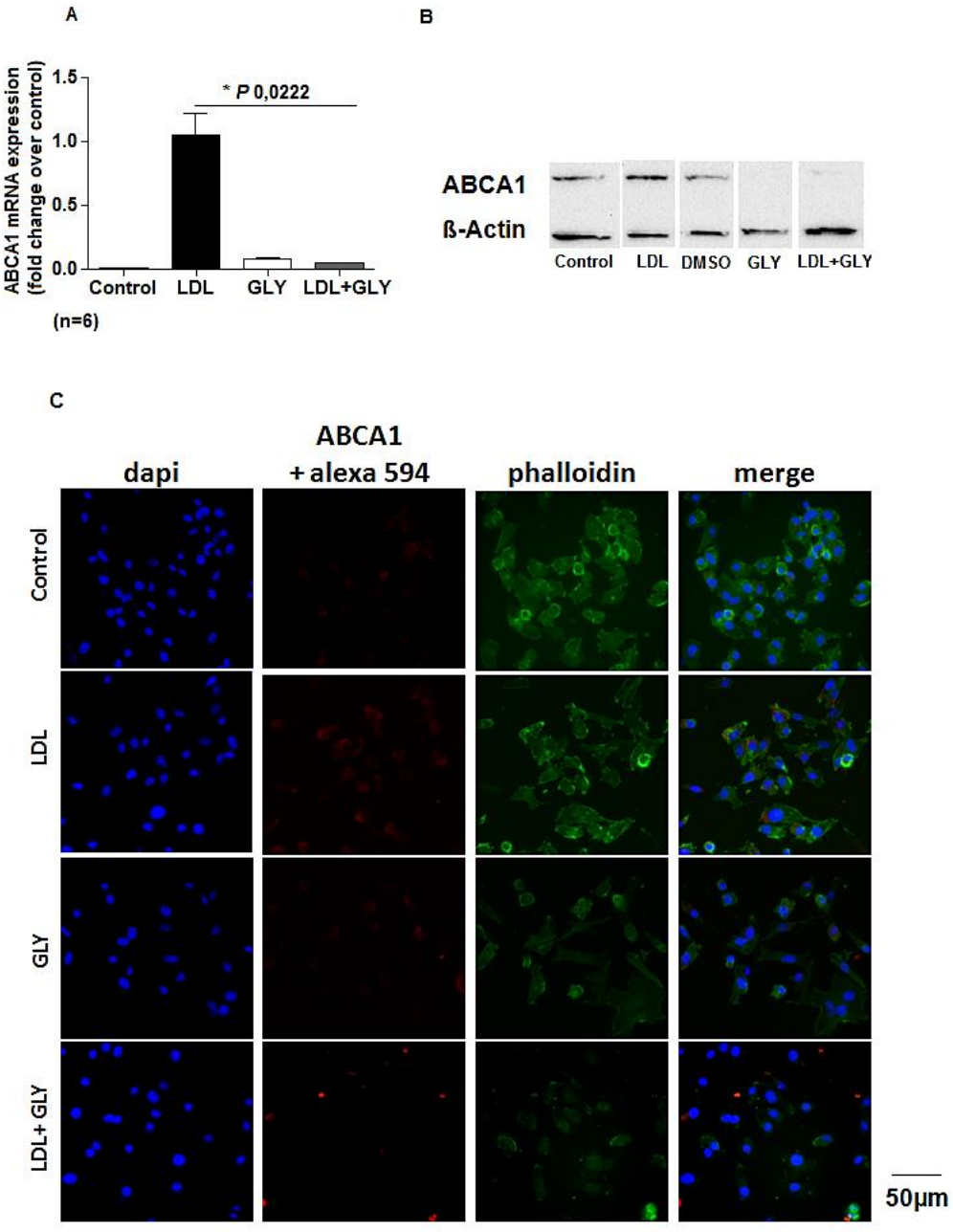
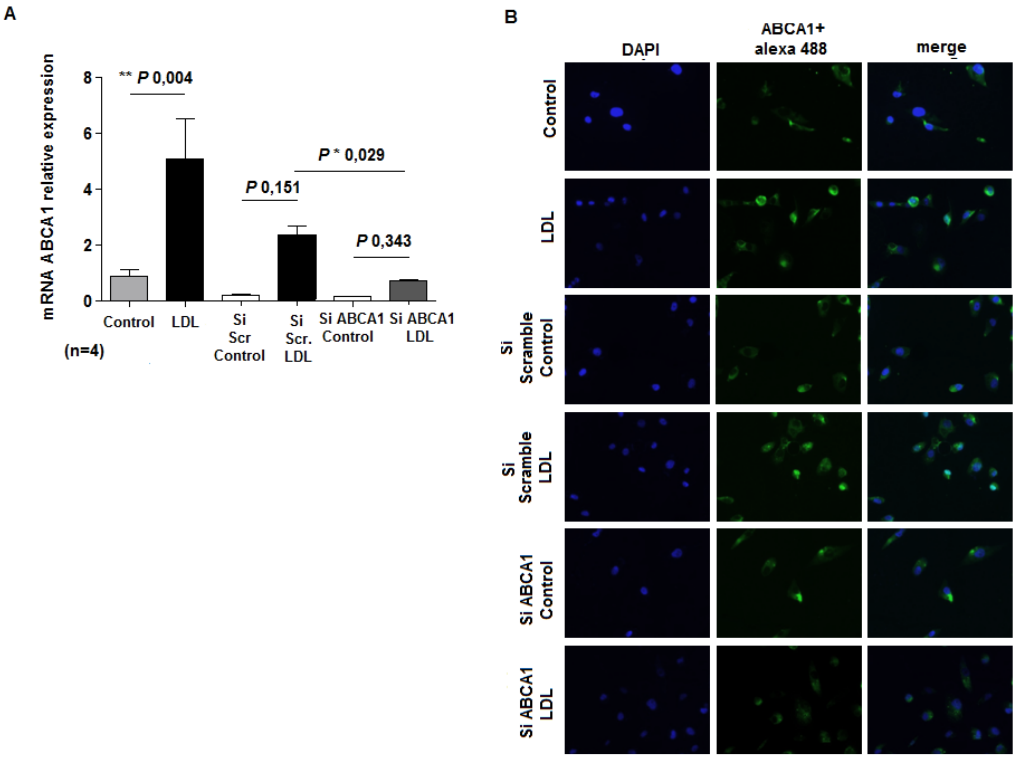


Figure 26: Glyburide decreases ABCA1 transcription and expression.

A. MDA MB 231 cells were cultured in LDL-C free and LDL-C enriched medium and treated with glyburide, for 48h. Total amount of ABCA1 mRNA was quantified by RT-PCR and proved to be overexpressed in cells cultured with 100ug/ml LDL-C compared to control, and decreased when these cells are treated with glyburide. **B** and **C** ABCA1 protein expression was detected by western blot and by immunofluorescence and results suggest a reduction of protein expression when cells were treated with glyburide. Immunofluorescence photos (X40). *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide.

siRNA ABCA1 inhibition prevents LDL-C-induced phenotype of breast cancer cells

Because the mechanisms of action glyburide are not completely understood, we wanted to be sure that the observed effect in tumor cells phenotype treated with glyburide is on the dependent of ABCA1 inhibition. So we specifically down regulated the ABCA1 gene expression by using small interfering RNA. Transfected cells exhibited down regulated ABCA1, confirmed by reduced ABCA1 mRNA (Figure 27 A) and protein expression (Figure 27 B). Functional tests showed reduction of proliferation rate (siScramble LDL 0,941±0,148 fold change over control vs siABCA1 LDL 1,774±0,251 over control, P 0,029) and migration (siScramble LDL 73,5%±9,678 vs siABCA1 LDL 47,24%±15,11, P 0,026) in cells siABCA1 transfected compared to those transfected with siScramble. Differences produced by siABCA1 are equivalent to those obtained with glyburide inhibition. We thus concluded that ABCA1 inhibition by glyburide is responsible for the phenotype modifications (Figure 27 C, D and E).



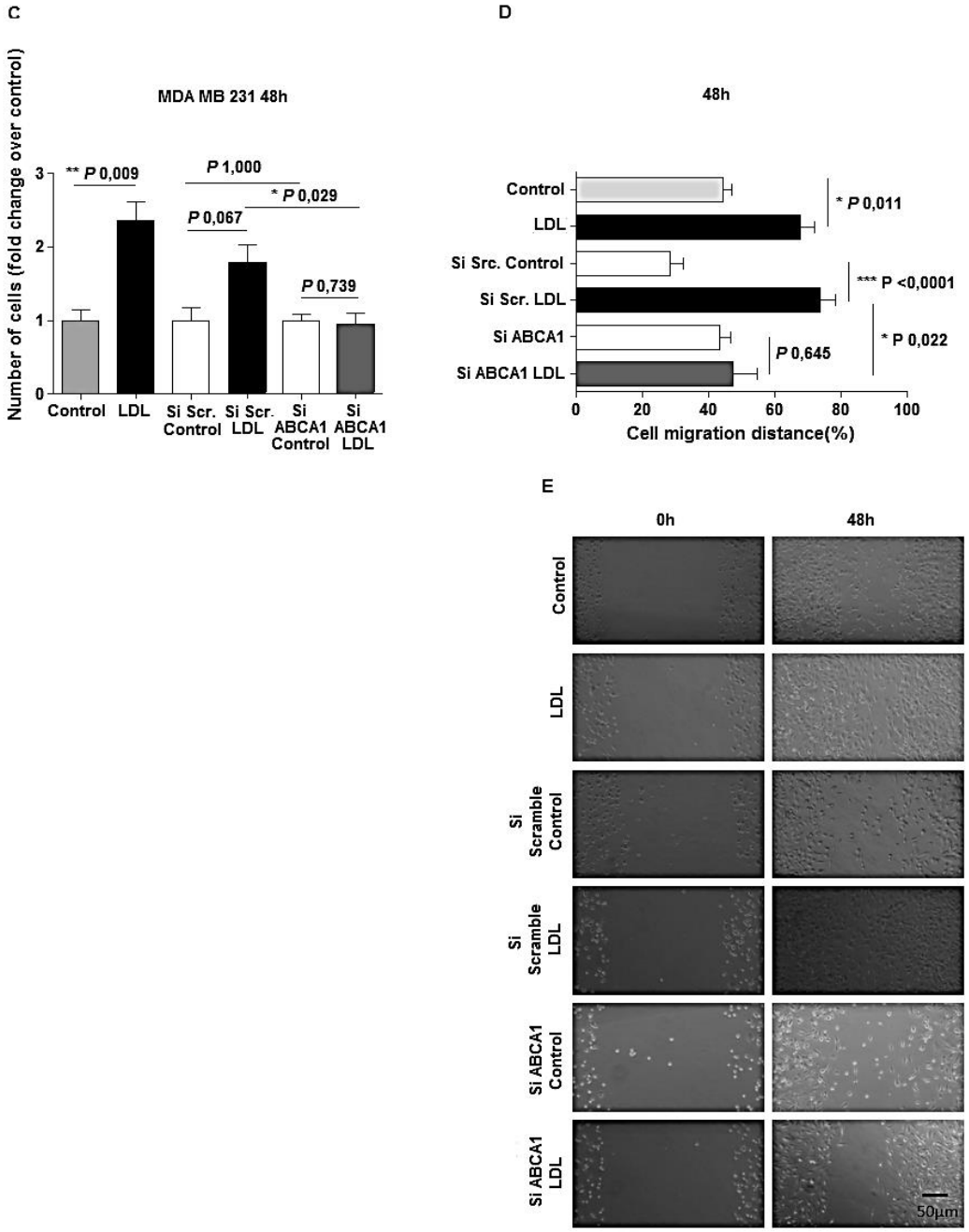


Figure 27: Down regulation of ABCA1 by siRNA specifically reduces LDL induced proliferation and migration of MDA MB 231 cells.

A Effect of siRNA on mABCA1 expression in MDA MB 231 was evaluated by RT-PCR. Cells transfected with siABCA1 have lower levels of mRNA compared to those transfected with scramble, especially when cultured in LDL-C enriched medium (*P* 0,029). B. Transfected cells and controls were grown on glass coverslips for immunochemistry staining. Representative photos (40 x magnifications) suggest ABCA1 protein down expression in siABCA1 transfected cells, growing in LDL-C enriched medium. **C.** After transfection, MDA MB 231 cells were cultured in LDL-C enriched medium or LDL-C free medium. After 48 h, the number of viable cells was evaluated by hemocytometer counts (at least 4 counts/well, in quadruplicates), after Trypan Blue test exclusion. Number of cells is expressed as fold change over control. **D and F.** After transfection, PC-3 cells were incubated with the medium containing 10% FBS for 24 h, and medium was switched to serum-free medium. After 24 h, cells were wounded and then cultured for 48 h with or without LDL-C (100 µg/mL). Cell migration into the wound was examined by phase-contrast microscopy (representative photos of experiments are shown with original magnification 100xC). *P* value and the number of the experiments are represented in the figure. Values are expressed as percentage of wound closure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein.

ABCA1 inhibition increases cellular cholesterol content

Knowing that ABCA1 acts mainly in cholesterol efflux, we wonder if the observed phenotype changes in glyburide treated cells were due to cholesterol homeostasis perturbation. In fact, we confirmed that ABCA1 blocking, either with glyburide or siRNA, leads to intracellular cholesterol accumulation (Figure 28 A and B). The cholesterol concentration in the culture medium was lower in the glyburide treated cells, although without statistically significant differences, corroborating the intracellular cholesterol accumulation effect of ABCA1 inhibition (Figure 28 C).

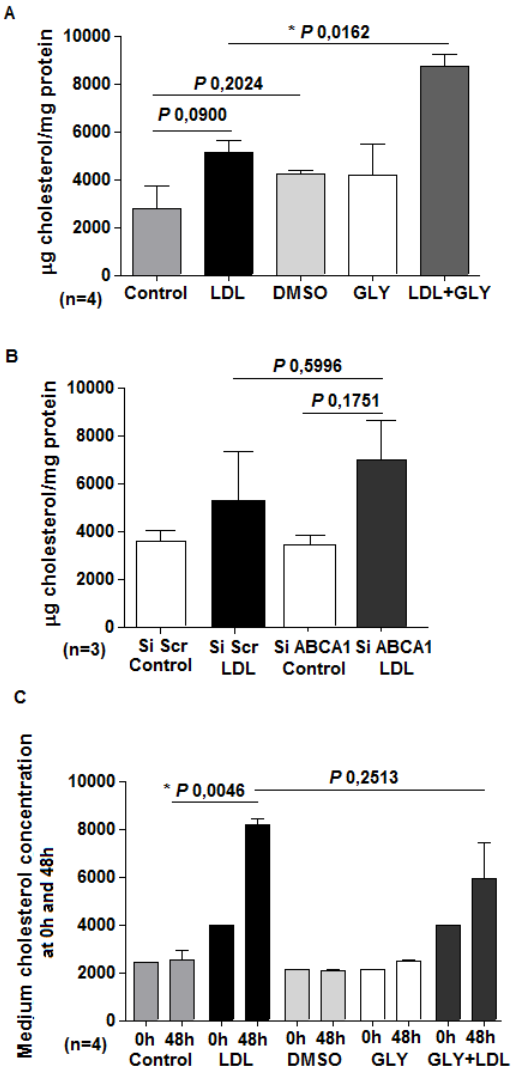


Figure 28: ABCA1 inhibition increases cellular cholesterol content.

A and **B**. Cholesterol content of breast cancer cells was measured by chomatography of cells lisates with use of Amplex® red cholesterol assay kit and showed an enrichement of LDL-C exposed cells compared to control cells, which is significantly higher when cells were treated with glyburide. **C.** The cholesterol concentration of cell culture medium was measured by the same method and revelead an increase in cholesterol content at 48h, in the LDL-C enriched conditions, although showing a modest decrease when cells were treated with glyburide. Note that in A and B, cholesterol concentration is given by cell, in C cholesterol concentration is given by culture sample. *P* value and the number of the experiments are represented in the figure. Values are expressed as percentage of wound closure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide.

According to the intracellular cholesterol accumulation hypothesis, HMGR and LDLR transcriptions are repressed in cells exposed to glyburide as well as in cells with ABCA1 silencing, both exposed to LDL-C (Figure 29).

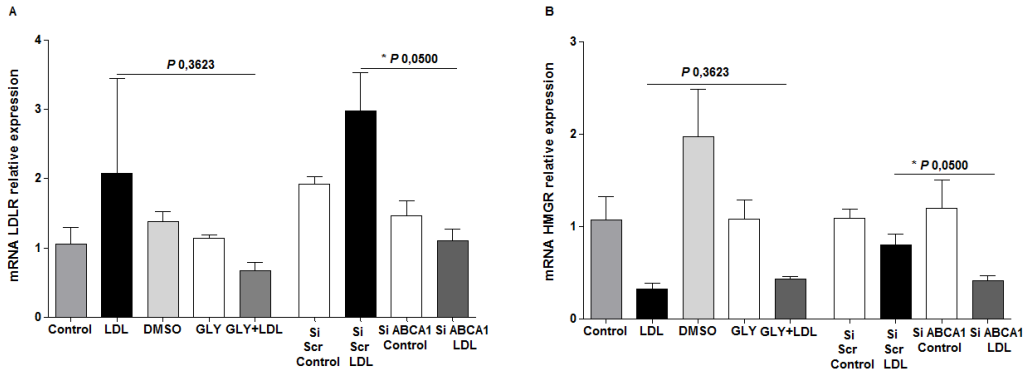


Figure 29:ABCA1 inhibition decreases LDLR and HMGR expression.

A and **B**. Effect of ABCA1 silencing on cellular cholesterol metabolism was measuring mRNA expression of LDLR and HMGR evaluated by RT-PCR. LDLR mRNA expression is enhanced by exogenous LDL-C availability but reduced when ABCA1 is inhibited. Conversely HMGR mRNA expression is decreased in the repressed in presence of LDL-C and do not change or is even reduced when ABCA1 is inhibited. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide.

Glyburide ABCA1 inhibition reduces ERK protein phosphorylation

We had previously demonstrated that cells death is not significantly increased when ABCA1 is inhibited, although apoptosis is slightly increased. Mechanistically, we tested the eventual modifications in proliferative cell signaling pathways and found a reduction in ERK phosphorylation upon ABCA1 inhibition, suggesting that ERK-cell signaling pathway may be mediating this effect (Figure 30).

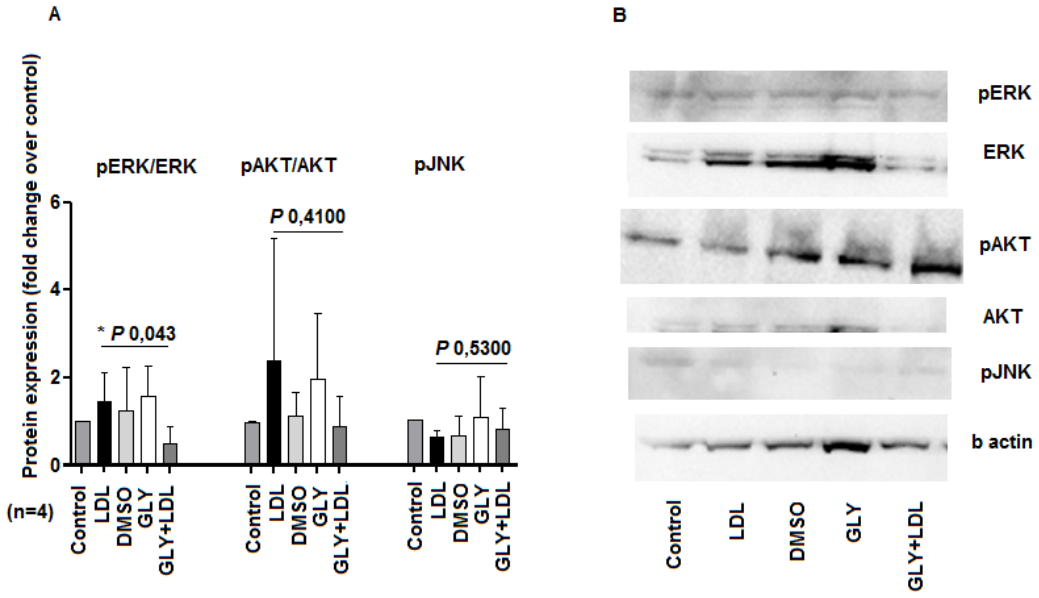


Figure 30: ABCA1 inhibition by glyburide reduces ERK protein phosphorylation.

A. MDA MB 231 cells were incubated with LDL-C free medium or LDL-C enriched medium and then treated or not with glyburide. After 24 h, whole cell lysate was analyzed by western blotting after treatment for 15 min (ERK1/2),30 min (Akt) and 10 min (pJNK). The graph shows the ratio of pERK1/2 to ERK1/2, pAkt to Akt and pJNK in each sample relative to βactin lane. **B.** Representative photos of western blot membranes are shown. P value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide.

In vivo, glyburide reduces tumor growth and lung metastasis incidence

We and others have previously shown that hypercholesterolemic diet induces breast cancer growth and increased incidence of metastasis[44,523].

In this experiment we used an immunocompetent mice model injected with mouse breast cancer cell line 4T1, exposed to hypercholesterolemic or normal diet. Test groups, treated with glyburide, show smaller tumors compared to non-treated groups, reaching statistically significance in the hypercholesterolemic fed mice (HD gly treated tumor volume mean is 0,82±0,126 fold lower than HD non treated group, P0,003). Glyburide treated groups also showed reduced incidence of lung metastasis when compared to non-treated groups (Figure 31 A and B). Lipid profile and glycemia, at the end of the trial, did not show differences between glyburide treated and non-treated groups (Figure 31 C and D).

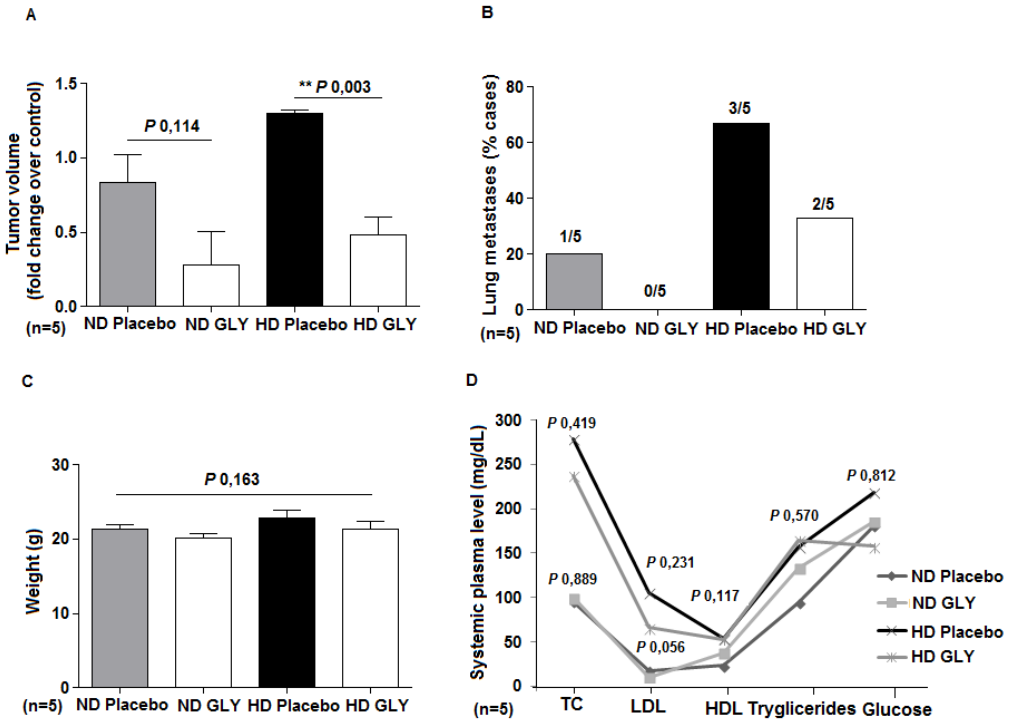


Figure 31: Glyburide reduces tumor growth and lung metastasis incidence.

A. BALB/c 4T1 mice model treated with glyburide (10mg/kg) show reduction of tumor size compared to non-treated mice, reaching significance in the HD fed group. **B**. BALB/c 4T1 mice HD fed show higher lung metastasis incidence which is decreased with glyburide treatment. **C**. Hypercholesterolemic diet (HD) fed mice have increased levels of low density lipoprotein (LDL-C) compared to normal diet (ND) fed mice but no significant differences to glyburide treated group. No significant differences in total cholesterol (TC), in high density lipoprotein (HDL), triglycerides and glucose levels were registered in HD compared ND, neither when treated with glyburide. **D**. Different diets or glyburide treatment did not produce significant weight differences. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein; GLY: Glyburide

Discussion

It is was previously shown that LDL-C induces phenotypic changes in breast cancer cell lines, characterized by increased proliferation, migration and loss of adhesion[523]. It is believed that tumor cells require cholesterol to proliferate, and those with the ability to up take and accumulate exogenous cholesterol would be in selective advantage, especially when the macroenvironment (systemic) is cholesterol-enriched. Accordingly, numerous studies have shown increased levels of cholesterol in tumors compared to normal tissue[531,532,533,534] and breast cancer cells expressing high levels of LDLR and ACAT-1 were shown to be more aggressive[411,288,535,536].

Interestingly, we found that LDL-C-selected cells abundantly express ABCA1 receptor. ABCA1 is the main ABC transporter responsible for the cellular efflux of free cholesterol and phospholipids[537,538,539].

ABCA1 is a protein belonging to a large family of conserved transmembrane proteins transporting a variety of substrates, including lipids, ions, amino acids, peptides, sugars, vitamins, steroid hormones, and drugs across cell membranes[540].

Although data on the expression and localization of cholesterol transporters in mammary tissues is scarce[541], ABCA1 expression was identified by immunohistochemistry in the epithelium of normal as well as in neoplastic human breast tissues[542]. ABCA1 overexpression was also reported in prostate cancer[543] and melanoma[544], and ABCA1 somatic mutations were implicated in colon cancer initiation[545]. Our data showed an overexpression of ABCA1 in breast tumors of patients with higher systemic LDL-C levels suggesting a modulation of ABCA1 tumor expression by the systemic host environment.

We hypothesized that for tumors growing in LDL-cholesterol-enrich macroenvironment; ABCA1 expression is a marker of cell dependency on exogenous cholesterol. ABCA1 is the mechanism by which these cells avoid intracellular cholesterol cytotoxicity and maintain the cholesterol-enriched microenvironment to the surrounding cells to proliferate.

In fact, block ABCA1 expression attenuates the LDL-cholesterol-induced phenotype. We demonstrated that ABCA1 inhibition reduces cell proliferation and migration, slightly increases apoptosis with no significant cell death. These changes go along with reduction of ERK phosphorylation exposing a modification of ABCA1 dependent cell signaling pathways.

Supporting our findings, Adlakha et al[546], recently demonstrate that ABCA1 is post-transcriptionally regulated by miRNA-128-2, a pro-apoptotic molecule, being the ABCA1 protein negatively expressed in the presence of this miRNA. In a mice trial, levels of miRNA-128-2 were reduced upon high-fat diet as compared with normal diet in several tissues. Conversely, mRNA levels of ABCA1 were increased. The authors speculate if diet modulates mi-RNA-128-2 expression, reducing pro-apoptotic stimulus and also leading to ABCA1 expression. In this case ABCA1 expression would have a protective effect against excessive cholesterol loading of endoplasmatic reticulum membrane that triggers unfolded protein response which eventually lead to the induction of apoptosis[547,548].

Conversely, Smith et al[290] interpreted their own results considering ABCA1 as an anti-cancer molecule. ABCA1 loss-of-function mutations lead to intracellular cholesterol accumulation and consequently to cholesterol metabolism instability and cancer initiation. However they do not considered different micro or macroenvironment, namely hypercholesterolemic environment in which the excess of cholesterol would be deleterious, driving the need of ABCA1. Taken together, results suggest that cholesterol metabolism is fundamental to cancer cells and ABCA1 is pivotal to cellular regulation.

Thereafter we intended to explore the mechanism by which ABCA1 would be involved in tumor aggressiveness.

To silence ABCA1 we used glyburide, a sulfonylurea family drug that inhibits ABC transporters[528,538]. Although the precise molecular mechanism of ABCA1 inhibition by glyburide is not known, we demonstrated the reduction of ABCA1 mRNA as well as ABCA1 protein (not quantified,) suggesting a transcriptional inhibition; and, in accordance, the specific inhibition of ABCA1 by siRNA interference produced exactly the same results. Then we used glyburide in all experiments to avoid transfection process, transient inhibition and to reproduce the ABCA1 inhibition in *in vivo* models. Others have also found an anti-tumor effect of glibenclamide, an analogue of glyburide, in *in vitro* experiments[549,550].

Our first hypothesis was that ABCA1 expression avoids excessive cholesterol accumulation. In fact ABCA1 inhibition leads to intracellular cholesterol accumulation, although cells repress LDLR and HMGR transcription, which is accompanied by cell proliferation and migration reduction. Concomitantly to intracellular cholesterol accumulation ABCA1 silencing reduces microenvironment cholesterol content and may decreases LDL-C signaling to neighbor cells to proliferate and invade. Nevertheless, we cannot exclude the fact that ABCA1 itself might act as a signaling molecule to surrounding cells, controlling their proliferation and consequently tumor growth.

On the other hand, ABCA1 is known to localize mainly in the plasma membrane. The translocation of phospholipids and cholesterol across the cell membranes implicates constant membrane and meso-domain organization modulating receptor-mediated signaling events, namely through lipid raft anchored proteins such as EGFR, ERBB2 or ER[551,291]. Inhibition of such movement, by inhibition of ABCA1 efflux may also explain reduction in cell proliferation and migration. In line, we already described a higher proportion of ERBB2 positive tumors in breast cancer patients with high levels of LDL-C at diagnosis[516]. It was as also shown that inhibition of cholesterol transport and ABCA1 degradation with serdemetan, a tryptamin compound with antiproliferative activity, reduces lipid raft signaling pathways in hematologic malignant cell lines[552].

Considering the importance of LDL-signaling to tumor aggressiveness we tested if the inhibition of LDLR expression, a major LDL-cholesterol acceptor, would avoid the LDL-cholesterol signal. However, transcriptionally reduction of LDLR did not replace the expected phenotype (Additional figure 1 and 2). A possible explanation is that others receptors, not studied may be involved, such as oxidized LDL receptor 1[553] or scavenger receptor class B type I[491]. Indeed, we and others[554,553,536] found high expression of other receptors-biological redundancy, in breast cancer cell lines exposing even more the relevance of cholesterol to tumor cells (data not shown). These findings turn the identification of ABCA1 role of uppermost importance, once it seems to be a more specific target to control cholesterol induced phenotype.

Although the mechanism by which ABCA1 potentiates tumor aggressiveness in cholesterol enriched environment and how its inhibition produces antitumor effect maybe complex, it confirms the importance of cholesterol metabolism in breast cancer and reveals ABCA1 as an important therapeutic target.

A mice model trial using glyburide showed that glyburide-treated mice have smaller tumors and lower incidence of lung metastasis, particularly in HD fed mice. We assume that *in vivo* glyburide effect occurs in cancer cells as in *in vitro*. Despite systemic effects, indirect effect on tumor cells, cannot be excluded, we showed that there were no differences in lipid profile, glycemia or mice weight at the end of the trial. Moreover, glyburide is a safe drug widely used in diabetes control [555].

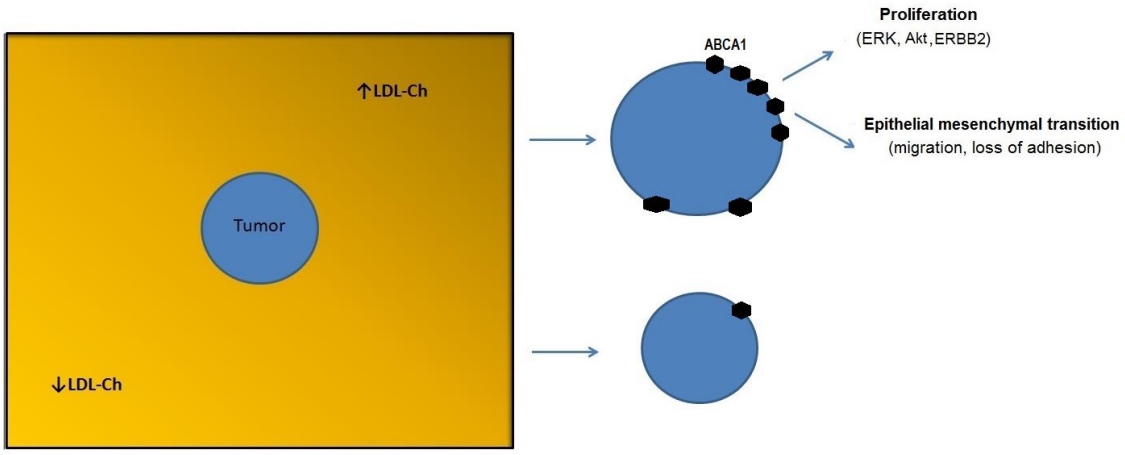


Figure 32: ABCA1 as a marker of LDL-C induced phenotype-Proposed Model

Patients with primary tumors with high ABCA1 score were seen to have more elevated LDL-C plasma levels. This fact points ABCA1 receptor as a marker of tumor aggressiveness and supports our hypothesis of LDL-C induced phenotype marker. The results from mice trial suggest an advantage in directly targeting this phenotype marker (Figure 32).

Conclusion

Results confirm the importance of cholesterol metabolism to breast cancer pathophysiology exposing the modulation of tumor behavior by the host systemic metabolism.

ABCA1 emerges as a cell marker of cholesterol dependency which can be explored as a biomarker as well as a specific therapeutic target.

Chapter 4

Plasma level of LDL-cholesterol, at diagnosis is a breast cancer prognostic factor [7]

Introduction

A tumor has been defined as a group of cells with autonomous capabilities able to survive, proliferate, develop neovascularization (angiogenic potential) and invade other organs (metastatic potential)[84].

Cancer cells metabolism has intriguing scientists for a long time (since Otto Warburg) but a little is known about this subject. Recently, reprogramation of cellular metabolism has been on the focus as an emerging hallmark of cancer, either as a way to accomplish the other demands of tumor cells or as the initial cause of the malignant transformation[556].

However, cancer cells living in an organism interact with the macrosystem and must be under the influence of systemic metabolism. In fact, some metabolic disorders have been linked to specific cancer behavior, including obesity[40,557] and diabetes[61]. On the other hand, the role of dyslipidemia, other common metabolic disorder, has generated great controversy, namely concerning to its impact on cancer incidence. Recently, we provided experimental[523] and clinical[516] evidence that the systemic cholesterol metabolism has not a neutral role in breast cancer pathophysiology. Data show that cells use the exogenous cholesterol to proliferate and undergo EMT and, according; patients with higher systemic LDL-C levels at diagnosis have larger and aggressive tumors. By prospectively following a cohort of patients with breast cancer diagnosed at stage I-III, we intended to validate that systemic cholesterol metabolism influences tumor behavior.

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[7] Results discussed in this chapter are published at Rodrigues dos Santos C, Fonseca I, Dias S, Mendes de Almeida JC. **Plasma level of LDL-cholesterol at diagnosis is a predictor factor of breast tumor progression**. *BMC Cancer*. 2014 Feb 26;14:132 (highly accessed paper).

Methods[8]

Study population and data collection

Study population and study design were previously described in chapter 1 and are briefly shown here (Figure 33). All women, who underwent for operable breast cancer at the Breast Unit of IPOLFG from January to December 2011, were prospectively assembled. Inclusion criteria: 1) invasive ductal carcinoma (currently named invasive carcinoma NOS[78]), confirmed by biopsy; 2) surgery as the first treatment with R0 resection; 3) informed consent. Exclusion criteria: 1) previous treatment (chemotherapy, radiotherapy, hormonotherapy); 2) hereditary breast cancer (confirmed by genetic analysis) or 3) taking lipid-lowering, anti-diabetic drugs (statins, fibrates, oral anti-diabetics, insulin) or corticosteroid in the previous year. The study was approved by the Ethics Committee of the IPOLFG.

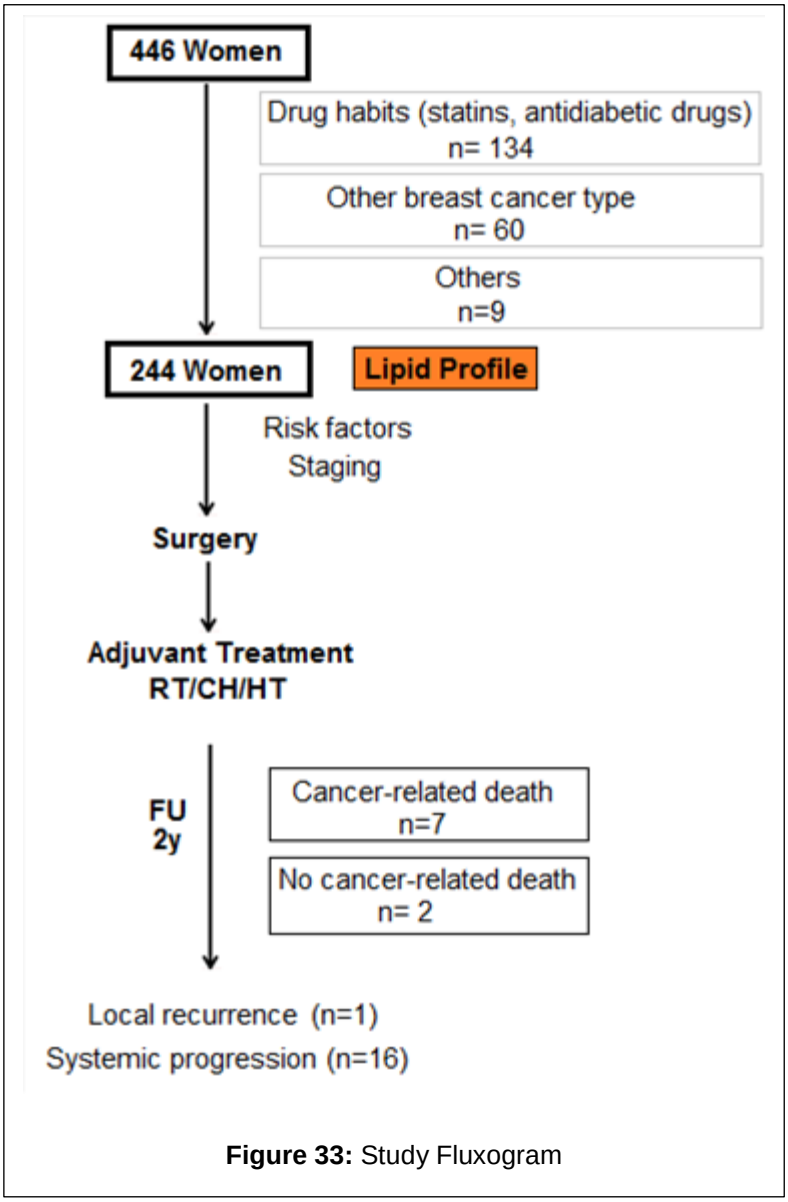

Figure 33: Study Fluxogram

Demography, risk factors and clinical examination were recorded in the first interview. Treatment was determined by the clinicopathological stage and patient characteristics according to the institutional protocols (following NCCN guidelines[492]), without changes related to the study (Additional

[8] The data collection was performed in collaboration with Breast Unit clinicians. The remainder tasks described in this chapter were performed by the candidate.

Table 2) . Fasting lipid profile was measured at diagnosis, along with routine preoperative exams.

Follow up, after surgery and adjuvant treatment (when appropriate), was scheduled every 6 months for 2 years and annual thereafter. Mammography was performed 1 year after surgery and then repeated yearly.

Biospecimen collection. Pathological and Immunohistochemistry assays

Fasting lipid profile was measured at diagnosis, along with routine preoperative exams as described earlier (Chapter 1).

Hormonal receptors were measured using standardized immunohistochemistry. HER2 was scored according to the WHO guidelines[493] from 0 to 3+. All cases with 2+ score were reevaluated using chromogenic *in situ* hybridation. Immunohistochemical staining for Ki67 was performed in a Dako Autostainer® (Dako, Glostrup, Denmark) using standard protocols, followed by counting positive cells in an automated cellular imaging system (ACIS® II, Dako, Glostrup, Denmark I).

Statistical analysis

Continuous variables are presented as mean (standard deviation) or median (interquartile range) if they have normal distribution or not, respectively. For categorical variables absolute values and frequencies are shown. Spearman rank correlations coefficients were calculated to examine correlations between continuous variables.

Patient subgroups were defined by LDL-C levels tertiles. Tumor characteristics (T stage, N Stage, immunohistochemical subtypes) and patient characteristics (BMI and age (by tertiles)) were studied as categorical variables.

Kaplan-Meier curves were used to determine overall survival (OS) and disease-free survival (DFS) rates with use of log rank tests. Cox proportional hazards models were used to estimate hazard ratios with 95% confidence intervals (CI), relating LDL-C level to DFS. Multivariate Cox model was adjusted to tumor T stage, N stage and subtype.

To assess the internal validity of our results we examined the association of lipid profile with BMI and age. The association of BMI and tumor characteristics, as well as OS and DFS adjusted to BMI were also determined.

For statistical purposes, cases were censored at the date of disease progression confirmation, death or at June 9th, 2013, whichever came first.

Likelihood ratio *P* values are reported to whole variables in the model. All *P* values are two-tailed.

The statistical analysis was done using IBM SPSS Statistics for Windows, Version 19.0(Armonk, NY: IBM Corp. Released 2010).

Results

During follow up, 1 woman had local disease relapse, 16 women had systemic tumor progression and 7 died. Other 2 women died of unconfirmed disease (Figure 33).

Survival and Cox Regression Model

At 25 months of follow up the DFS in LDL T1, LDL T2 and LDL T3 groups was 100%, 90,6% and 88,3%, respectively (log rank test 0,013) (Figure 34 A). OS had no statistically significant differences between LDL-C tertiles groups (Figure 34 B).

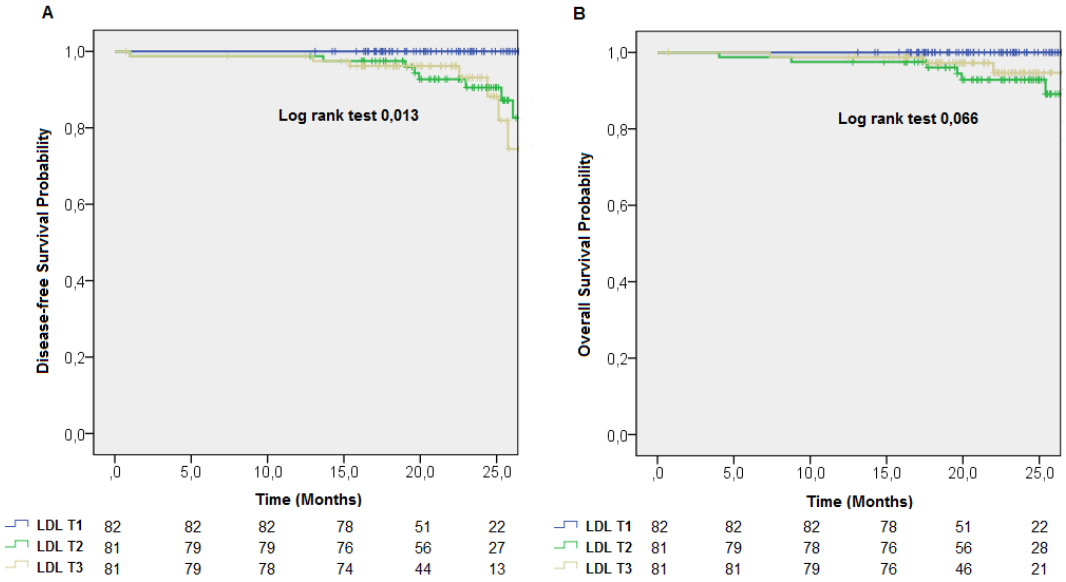

Figure 34: Overall and disease-free survival in LDL-C tertiles groups. Kaplan–Meier curves.

A. At 25 months, overall survival is 100 % in LDL T1, 92,8% in LDL T2 and 97,2% in LDL T3 (Log rank test *P* 0,066). **B**. At 25 months, disease-free survival is 100% in LDL T1, 90,6% in LDL T2 and 88,3% in LDL T3 (Log rank test 0,013).

The association of BMI and tumor characteristics, as well as OS and DFS adjusted to BMI were also determined and differences of DFS across LDL-C tertiles still significant (Figure 35).

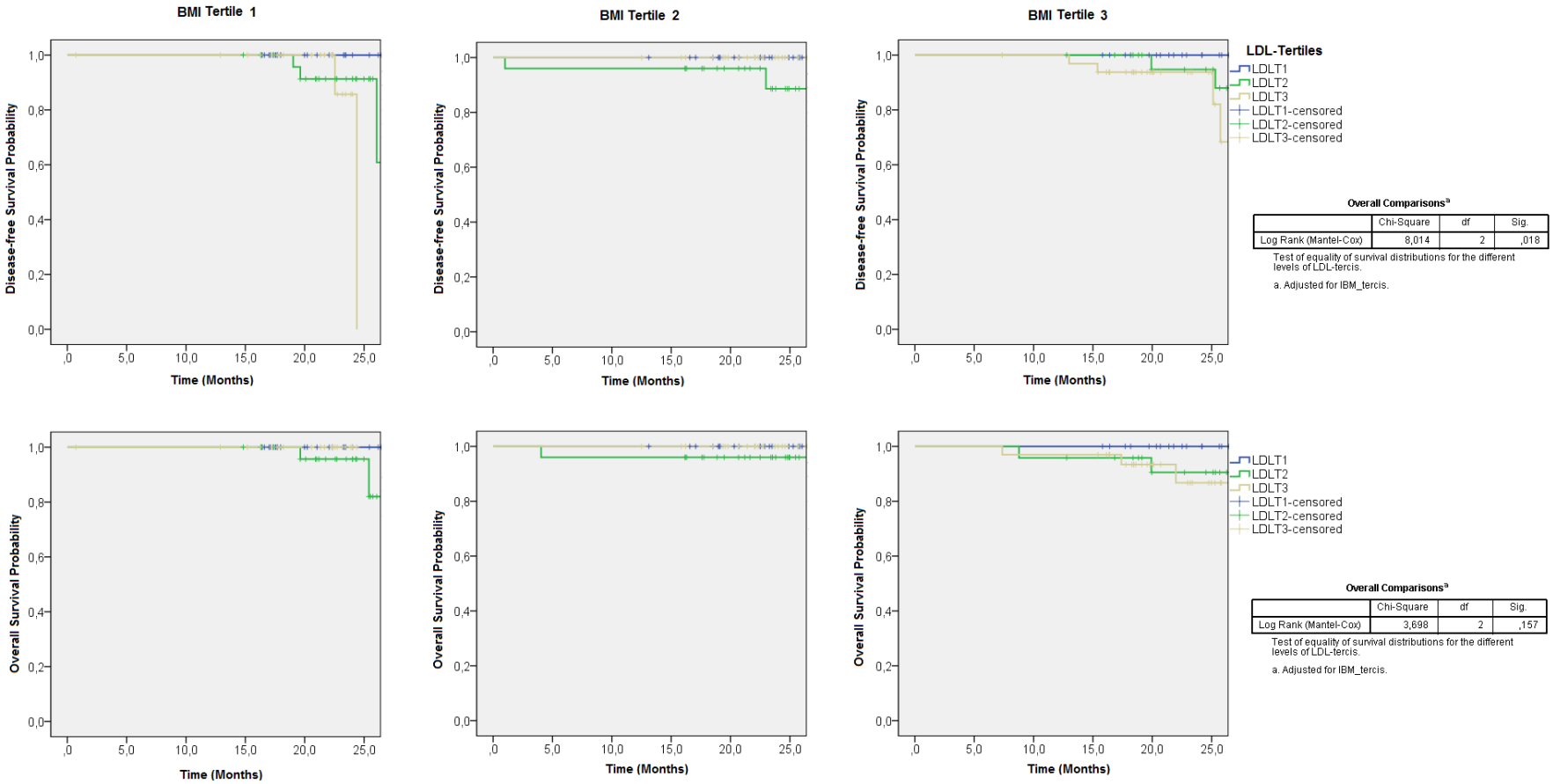

Figure 35: Overall and disease-free survival in LDL-C tertiles groups adjusted to **BMI**

Kaplan–Meier Curves for overall survival and disease free survival to each BMI tertile are shown. At 25 months of follow up disease-free survival shows differences across LDL-C tertiles, BMI adjusted (Log rank test *P* 0,018). But overall survival is not different in LDL-C tertiles (Log rank test 0,157).

Cox regression model to DFS, adjusted to tumor T and N stages, and breast cancer immunohistochemical subtypes, revealed that LDL-C≥117 mg/dL, at diagnosis, is associated with poor disease-free survival (HR 0,129; CI 0,017-0, 978, *P* 0,048)(Table 10).

Table 10: Cox multivariate regression model for disease-free survival

Variable	*HR*	*95% CI*	*P value*
Tumor T Stage (≥T2)	0,822	0,264-2,565	0,736
Tumor N Stage (≥N1)	0,551	0,201-1,515	0,248
LDL-C (> 117 mg/dL)	0,129	0,017-0,978	0,048
Luminal A (yes)	0,599	0,072-5,017	0,637
Luminal B (yes)	0,532	0,047-6,026	0,610
Triple Negative (yes)	0,128	0,015-1,111	0,062
HER2 type (yes) [a]	--	--	--

a. Degree of freedom reduced because of constant or linearly dependent covariates
LDL-C: Low Density Lipoprotein; T and N: TNM stages; HR: hazard ratio, CI: confidence interval

Discussion

All of the women included in the study were treated with curative (R0) surgery plus radiotherapy and or systemic therapy according to NCCN guidelines[492].

Curative surgery means that all macroscopic tumor (with clear margins) and clinical metastatic lymph nodes were removed. Radiotherapy had been shown to reduce local recurrence, and very recently was demonstrated to have also impact in late overall survival[558]. Chemotherapy and hormonotherapy have proved impact on survival[559] because are expected to eliminate distant subclinical micrometastasis.

We previously shown that aggressive tumors were more common in patients with high LDL-C levels at diagnosis[560] and here we demonstrate that these patients also have reduced DFS at 2 years of follow-up. LDL-C level appeared as an independent factor associated with worse prognosis, besides classical prognostic factors or tumor subtype.

For operable breast cancer, 2 years of follow up may be considered short, but is also reported that disease relapse has a peak of incidence in the first 2 years after diagnosis[561,562]. As a limitation we could not avoid the possible influence of adjuvant treatment either in lipid profile or disease progression, once no modifications to the routine protocols were introduced. Nevertheless the strong association of LDL-C level and tumor size before treatment favors the LDL-C as a putative prognostic biomarker. It is also not possible to exclude the common association of cholesterol levels, obesity and the

variance in health awareness before diagnosis. However results were adjusted to BMI and, during follow up, all women were evolved in the same program of surveillance and health control.

Elevated LDL-C is a well established cardiovascular risk factor but the significative differences in DFS of LDL-C tertiles groups and not OS, which would include cardiovascular causes of death, supports the independent risk for tumor-related event. Moreover, the adjustment to BMI, still show differences in DFS across LDL-C tertiles groups.

The longitudinal correlation with LDL-C level at diagnosis favors a primordial selection; however the continuous effect of elevated LDL-C on micrometastasis could not be excluded because lipid profile was not systematically accessed after breast cancer treatment. The different metabolic patterns of the primary tumors found in chapter 3 supports that cancer cells are not independent of the macrosystem and that the modulation of systemic metabolism could have impact on tumor cell metabolism and behavior: high systemic LDL-C levels may select for aggressive clones.

On the other hand, cholesterol could also promotes other systemic alterations favoring tumor dissemination, independently of the tumor cells malignant potential, such as local or systemic inflammation, endothelium permeabilization, lymphatic concentration and flux[563,564].

Results expose that cholesterol metabolism, and specifically LDL-C levels, influence tumor behavior being a prognostic biomarker and an accessible therapeutic target.

Being hypercholesterolemia a global prevalent feature, measurable and treatable, its management in breast cancer would have a major impact. Taken together, results strongly supports that plasma cholesterol levels must be evaluated and controlled in breast cancer patients and their effect should be analyzed in clinical protocols.

Conclusion

In conclusion, results expose that cholesterol metabolism, and specifically LDL-C levels, influence tumor behavior and is a prognostic biomarker and an accessible therapeutic target.

Final Discussion

Our working hypothesis in this thesis was that a tumor is not an independent entity in an organism. Rather, it influences and must be influenced by the systemic (metabolic) environment.

Despite the assumption that cachexia is a consequence of tumor spread and its metabolic needs, very little is known about the relationship of systemic metabolism and tumor behavior. Clinical and epidemiological studies have been providing evidence that obesity and diabetes, common metabolic features of Western societies, are associated with higher incidence and worse cancer prognosis. Some metabolic environments may potentiate tumor development, by acting directly on tumor cells or by promoting systemic modifications that favors tumor spread or reduce the host ability to antitumor defense.

Although dyslipidemia, namely hypercholesterolemia, is also extremely prevalent, the role of systemic lipid metabolism in cancer is unclear and is still under intense scrutiny.

Lipids are essential for vital cell functions, and therefore many steps of lipid metabolism, have been conserved over time and across the species. It is expected that tumor cells also make use of this well established property in nature.

For these reasons, we wanted to know how the high prevalence of dyslipidemia correlates with tumor pathophysiology and how its control can change the natural history of the disease, using breast cancer as a model. Despite improvements in detection and treatment, it is the most common tumor in women remaining one of the most important causes of death worldwide. Moreover, several aspects of breast cancer biology, suggest a putative dependency of lipid metabolism. Breast epithelial cells themselves are dependent on the absorption of lipids from circulation for milk production; the majority of breast cancers are stimulated by steroid hormones, which are cholesterol metabolism derived; breast cancer is highly prevalent in regions with high incidence of dyslipidemia and interestingly we found an overlap in the pattern of global distribution of breast cancer and cholesterol levels in the population.

Hence, the central question of this thesis was to study the role of systemic cholesterol metabolism in breast cancer development.

First, we reviewed the literature and observed that

1) Epidemiological studies on breast cancer incidence and dyslipidemia are contradictory and although first reports associate cancer with low levels of cholesterol, this association is today accepted as a reversal causation. It means that the low levels detected in circulation reflect cholesterol consumption by the tumor. The unequivocal evidence of dyslipidemia as a risk factor for breast cancer similarly to what was shown to cardiovascular diseases, is very unlikely to be achieved by human studies, namely because of the long time course for tumor development.

2) Laboratory (in *vitro*/ *in vivo*) findings support the causal influence of cholesterol in breast cancer pathogenesis. However, it should be noted that *in vivo* studies are very recent and were not available at the beginning of this project.

3) Knowing that statins are commonly used to hypercholesterolemia control, we searched for evidence of the effect of lipid lowering drugs in breast cancer incidence. We concluded that studies were designed to assess cardiovascular endpoints and conclusions to oncologic measures are scarce. Nevertheless, ongoing trials on breast cancer prevention with statins should add some new and important information.

4) Breast cancer patients usually have altered lipid profiles and modifications in that profile occur during different tumor stages, suggesting that the interaction of the tumor with the host systemic lipid metabolism is not constant over time.

Thus, the available data indicate that the metabolism of systemic cholesterol influences breast cancer pathophysiology. Being a causal relationship so hard to demonstrate because of the need for lengthy studies and the impossibility to control all variables that interfere with the lipid profile over this period, we tried to focus on the role of cholesterol in breast tumor progression.

The widespread use of screening mammography allowed the identification of subclinical tumor lesions considered early breast tumors. In clinical terms (human) this is the earliest tumor stage we can identify to study the natural history of the disease. So, we designed a prospective study including women with early breast tumor, with the most frequent histologic type (invasive carcinoma, NOS) and determine the lipid profile at diagnosis and before any therapy that could modify tumor characteristics. We also controlled the intake of drugs that could modify the lipid profile, including statins, fibrates, insulin, oral antidiabetics or corticosteroids. Likewise we controlled the individual variables which could interfere with lipid profile such as age, parity, menopausal status and BMI. Initial analysis clearly shows that LDL-C, and no other lipid profile variables, correlates with tumor size

and patients with high LDL-C levels have more aggressive tumors. Meaning these tumors have classical worse prognosis characteristics: high proliferative index, less differentiated and higher prevalence of HER2 positive tumors.

These findings, together with the fact that the most common dyslipidemia type is high LDL-C with low HDL-C, lead us to go further on the understanding of the LDL-C signaling mechanisms.

In vitro exposure of breast cancer cells to exogenous LDL-C enriched medium intended to mimic systemic LDL-C influence on tumor cells. We initially used increasing LDL-C concentrations and observed a dose dependent effect until a noxious level (data not shown) and subsequently reproduced all the experiments with a concentration of 100µg/ml, considering that interstitial LDL-C concentration is approximately 1/10 of LDL-C plasma concentration[565].

The cells exposed to higher concentrations of LDL-C developed a more aggressive phenotype, characterized by increased proliferative and invasive capacity; in agreement with the findings of the clinical study. Gene expression analysis of these cells revealed that exposure to LDL-C also induces genotypic changes. In other words, the more aggressive behavior of these cells seems to be induced by increased transcription of genes involved in cell proliferation and survival. It was also demonstrated the activation of cell signaling pathways related to cell proliferation and survival as Akt and ERK.

To further corroborate these results, we used breast tumor orthotopic animal models (with different backgrounds and different types of cell lines) to demonstrate that dyslipidemia induced by cholesterol-enriched diet also recreates a phenotypic expression of increased aggressiveness in breast tumors.

Although the clinical association of higher LDL-C levels and more advanced and aggressive tumors does not exclude a correlation of reverse causality; meaning that the highest serum LDL-C levels may correspond to a reaction to larger and more proliferative tumors rather than to be the cause of these phenotypes; studies *in vitro* and *in vivo* strongly support the hypothesis of LDL-C induces aggressive phenotype. These data also favor the notion that tumors are not metabolically independent of the organism and the modulation of the systemic environment can be used for prevention and treatment of breast tumors.

After showing that systemic LDL-C promotes breast cancer progression, it was critical to understand how to prevent such effect.

Thus, the most direct way to reduce this worse prognosis phenotype would be to prevent dyslipidemia and or to treat dyslipidemia when diagnosed. The most widely used drugs in the treatment of hypercholesterolemia are statins. As an exploratory strategy of this approach to breast cancer we conducted a pilot trial with simvastatin in animal model. However, one problem in pre-clinical evaluation of statins is precisely the inability to reduce cholesterol levels in animals commonly used as models such as mouse. Therefore, despite the trend towards smaller tumors in the group treated with statins, this cannot be associated with lowered levels of LDL-C. It is known that statins can have side effects with benefit and in this case with an impact on tumor growth, such as the anti-inflammatory properties. But we must also consider that the effect of statins may occur either in the liver with consequent reduction in systemic cholesterol levels, and in tumor cells themselves, leading to reduced synthesis of cholesterol and all pro-proliferative products derived from the mevalonate pathway (as demonstrated recently in *in vitro* and preliminary clinical results). Nelson et al[342] also described a positive effect of atorvastatin in breast tumors volume in mice models, but do not show the cholesterol measurements.

Furthermore, the threshold for considering dyslipidemia diagnosis and tretament values are widely studied for the prevention of cardiovascular events, but nothing is known about the limit values for cancer events. This was the reason that led us to not consider a threshold value for dyslipidemia in the study group (considering the cardiovascular limits, 80% of women with breast cancer were advised to lipid lowering treatment) but rather a relative subgroup analysis which resulted in the demonstration of differential effects with higher values compared to the lower. Specifically, we found differences in behavior of the tumor to LDL-C levels greater than 117mg/dl. However tumor response to LDL-C levels is not straight forward, i.e. above very high LDL-C levels tumor agressiveness was not increasing, leading to assume that there is a saturation point value above which cell response no longer exist. Another possible explanation is that very high LDL-C level is harmful to cells whose cholesterol metabolism depends on exogenous supply (such as observed *in vitro*) and again the systemic metabolic environment selects tumors with different metabolic profiles attenuating the phenotypic expression of aggressiveness.

This dependency on the exogenous supply was also our main interpretation for the overexpression of cholesterol exporters observed in the gene expression (microarrays) analysis.

The high expression of ABCA1 (the main exporter of intracellular free cholesterol) in tumors exposed to high concentrations of LDL-C was one of the most intriguing findings.

For proliferative cells with high demanding of cholesterol and well adapted to a medium enriched in cholesterol, dispense of such a metabolite seems to be counterintuitive. We considered as the most plausible interpretation that this was a defense mechanism for cells that depend on the external environment to avoid excessive accumulation of cholesterol. We had previously found that too much cholesterol was toxic. However, while the inhibition of cholesterol export promoted cellular accumulation and reduced cell proliferation, this did not promote significant cell death.

It was recognized (namely by the pivoteal contribution of Otto Warburg) that tumor cells even in the presence of oxygen reprogram the metabolism of glucose for less apparently efficient way of producing energy, the so-called "aerobic glycolysis" in which only 4 ATP molecules are generated per glucose molecule, instead of the 36 resulting from the oxidative phosphorylation. This process also leads to the production of lactic acid, which has been incriminated in the promotion of carcinogenesis. Some authors have identified various tumor cell populations with different metabolic needs namely with preference for lactic acid, and this metabolic switch is interpreted as a form of symbiosis between different tumor populations (Warburg effect).

Similarly it is possible that cholesterol export to the interstitial space is a way of perpetuate the LDL-C signal induced to the surrounding cells. Similarly to the ability to incorporate glucose which has been used to clinically detect metabolic activity of tumor cells through the PET scan, the ability to incorporate LDL-C may also be useful for cancer detection and ABCA1 may serves as a marker of this metabolic feature and an approachable target to stop cancer cell communication.

As an obvious way to stop this communication we prevented the internalization of LDL-C through the LDLR, which had no significant impact on the phenotype LDL-C induced. Although LDLR is known to be the main LDL-C receptor, there are others receptors documented, as SRBI or OLR that might keep the amount of cholesterol and communication. In any case from the systemic point of view, the blockage of LDLR would lead to an accumulation of circulating LDL-C with atherosclerotic consequences. The other way to stop this communication would be to block signaling through inhibition of ABCA1, which reduced cell proliferation and invasiveness. ABCA1 inhibition appears relatively specific in this chain and a better therapeutic target. We also had the opportunity to demonstrate that tumors of patients with high levels of LDL-C have overexpression of ABCA1, supporting the ABCA1 as biomarker of exogenous cholesterol dependency and a potential therapeutic target.

Blockage of this receptor was obtained *in vitro* and *in vivo* with a drug which pharmacokinetic and pharmacodynamics profile is well known in clinical practice – glyburide (glibenclamide family). Even though in a pre clinical setting, this is an important contribution to the expansion of therapeutic armamentarium to breast cancer. The chemoprevention with other oral antidiabetic (metformin) is also in advanced stages of clinical studies and apparently with good results. The mechanism of action of glibenclamide is different from the mTOR pathway inhibition elicited by metformin and emerges as another potential and accessible therapeutic tool. Furthermore, statins by reducing HMGR and thus the intracellular concentration of cholesterol has the potential to also inhibit the expression of ABCA1 and may constitute another parallel benefit of statins, independent of plasma cholesterol levels. This potential action, provide additional support to ABCA1 inhibition.

Like us, other groups recently identified ABCA1 as a player in breast cancer. Supporting our findings, Adlakha et al[546], demonstrate that ABCA1 is post-transcriptionally regulated by miRNA-128-2, a pro-apoptotic molecule, being the ABCA1 protein negatively expressed in the presence of the miRNA. In a mice trial, levels of miRNA-128-2 were reduced upon high-fat diet as compared with normal diet in several tissues. Conversely, mRNA levels of ABCA1 were increased. The authors speculate if diet modulates miRNA-128-2 expression, reducing pro-apoptotic stimulus and also leading to ABCA1 expression. In this case ABCA1 expression would have a protective effect against excessive cholesterol loading of endoplasmic reticulum membrane that triggers unfolded protein response which eventually lead to the induction of apoptosis[547,548]. Smith et al[290] found that ABCA1 loss-of-function mutations lead to intracellular cholesterol accumulation which favors cancer initiation and considered ABCA1 as an anti-cancer molecule. Even though apparently contradictory to our observations, these findings support that cholesterol import or intracellular enhanced biosynthesis are pro-cancerigenous and so corroborates our data. The authors do not explored the setting of excessive cholesterol accumulation, due to ABCA1 loss of function, which would have the opposite outcome. Nevertheless, reaffirms that ABCA1 is an important and relatively specific player in the fundamental cholesterol metabolism of cancer cells.

Particularly important in our studies was the follow-up of a population of patients with early breast tumors. At 2 years follow-up was possible to demonstrate that the levels of LDL-C at diagnosis correlated with tumor size at presentation and is an independent prognostic factor for DFS.

Several limitations should be considered in this analysis as the criteria for selection, the time of observation, adjuvant therapy impact, and possible changes in lipid profile.

Because of selection criteria, only patients with initial surgical treatment were included. Knowing that triple negative tumors and HER2 type tumors are generally larger and have greater probability of lymph node metastases at diagnosis are more likely referred for neoadjuvant chemotherapy which led to an underrepresentation of these tumor types in the population. Although we had not found differences in lipid profile between different tumor types in the analysis of the population and a recent article demonstrating indirect activation of ER by cholesterol metabolites[342], our experimental results show a greater effect of cholesterol availability on ER negative cells (MDA MB 231 and HTB 126 versus HTB 20). ER positive and ER negative cell lines seem to have differential lipid metabolism and needs and it is recognized that ER positive cells have impaired cholesterol biosynthesis compared to ER negative.

Wang et al [283] found that differential expression of lipid metabolism genes may be involved in the risk for subtypes of breast cancer and are expressed at higher levels in the contralateral breast of ER negative cases leading to potential biomarkers to ER negative breast cancers. So the exogenous lipid dependency may be more crucial to RE negative cells. This is especially important because ER negative cancers have less therapeutic options. The demonstration that lipid metabolism modulation, namely hypercholesterolemia control would help in ER negative breast cancer prevention and treatment is a finding with expected high impact.

In the natural history of operable breast tumors 2-year follow-up is too short, knowing that these are tumors with a very good prognosis, with a low rate of events and late recurrence (up to over 20 years). However, is also recognized that there is a peak of recurrence in this period and we can conclude that at least for early recurrence / progression there is a strong association with LDL-C levels in the diagnosis.

Patients were treated according to the institution protocol and therefore subject to local adjuvant (radiotherapy) and systemic (chemotherapy/ hormonotherapy) therapy. Especially the latter is recognized to have impact on survival and this effect could not be excluded from the analysis. Furthermore, is known that tamoxifen and aromatase inhibitors interfere with lipid profile and even contribute for the control of dyslipidemia. One can speculate how the positive effect of its action also depends on this lipid profile modulation. Strictly speaking, a prognosis factor can only be validated in a population without other interventions; however it is not acceptable to deny the benefit of current

therapeutics to test new prognostic factors. Instead, they must be evaluated in the current treatment scenario.

Nevertheless, even after the adjustment to important factors as tumor intrinsic prognostic markers and BMI, plasmatic LDL-C level at diagnosis still predictive of reduced DFS.

From biological point of view these data strengthen the hypothesis that systemic metabolic environment in early stages of tumor development can select tumor characteristics. The immunohistochemical evaluation of a pilot sample to the key points of intracellular cholesterol metabolism *in vivo,* further corroborate this hypothesis suggesting the adaptation of the tumor to the environment that we had observed *in vitro*, including the overexpression of ABCA1 in cases of tumors grown in enriched-cholesterol environments .

Despite this apparently direct effect of systemic metabolism of cholesterol in the primary tumor, we cannot eliminate the possible systemic influences of hypercholesterolemia in the body, which can also be facilitators of tumor progression and dissemination.

Together, results strongly support an effect of systemic LDL-C in breast tumor progression and ABCA1 expression appears as a marker of tumor responsiveness to systemic LDL-C. This observation recalls the effect of systemic estrogen. Similarly the reduction in estrogen levels (e.g. ovarian castration or with aromatase inhibitors) or blocking of specific hormone receptors (e.g. tamoxifen) inhibits this proliferative effect, also the control of LDL-C, or blocking cellular effector molecules may prevent its impact and need to be exploited clinically.

In summary the results lead us to propose as the final model that breast tumors require cholesterol availability to proliferate and progress. If these tumors develop in a cholesterol-enriched environment, the well adapted tumors will be selected and dominate. The contribution of other cholesterol effects in the body for the enhancement of the progression and spread of tumors is still under evaluation.

Results also support and impose a trial to test the effect of hypercholesterolemia control in breast cancer patients.

Importance of the findings and future perspectives

The importance of our findings can be considered at two levels. The first, in a biological point of view, reveals that one tumor is not a collection of independent cells in the body. Conversely, it modifies the organism leading to overall degradation, but is primarily influenced by the host characteristics. Although this influence is often neglected, mainly because of the difficulty studying this issue, it likely extends to all dimensions of the body. Here we showed how systemic metabolism features and specifically, host cholesterol metabolism may influence tumor progression. Second, and extremely relevant, is the demonstration that a highly prevalent condition such as hypercholesterolemia may be among the risk factors for the progression of the most common and high mortality associated cancer in women. For prevalent pathologies, a risk or worsening factor even though not major has a giant scale effect. Presently, nobody doubts of the connection between smoking and cancer, but obesity and all its comorbidities are starting to replace tobacco as the main risk factor for malignancy. The demonstration of a correlation between two diseases as prevalent as breast cancer and dyslipidemia provides important information for the management of public health data.

For now, our results clearly implicate cholesterol in the pathophysiology of breast cancer; disclose a predictive value of LDL-C level at diagnosis and expose the need to control hypercholesterolemia in women with breast cancer, once women with elevated LDL-C levels have a higher risk of cancer progression and cancer-related death.

As future prospectives it is mandatory to conduct a clinical trial to evaluate the effect of the control of hypercholesterolemia in patients with breast cancer as a secondary prevention strategy. In parallel, more models must be developed (exprimentals and clinical) to clarify the role of cholesterol in tumor initiation and to understand if there is also a role for cholesterol control in primary prevention.

Not of less importance and presently ongoing is the study of the effects of systemic metabolism in the organism, namely by producing changes that enhance tumor spread or metastasis in target organs.

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Additional Tables

Additional Table 1: Univariate logistic regression to the risk of tumor Size ≥20mm

Variable	*Univariate Analysis*			N
	HR	*95% CI*	*P value*	
Total Cholesterol T1	0,491	0,285-0,845	0,010	244
Total Cholesterol T≥2	1,912	1,113-3,285	0,018	244
Total Cholesterol T3	1,489	0,869-2,550	0,146	244
Total Cholesterol Q1	0,555	0,312-0,070	0,081	244
Total Cholesterol Q4	1,351	0,763-2,395	0,301	244
HDL-C T1	1,458	0,849-2,504	0,171	241
HDL-C T≥2	0,686	0,399-1,178	0,171	241
HDL-C T3	0,739	0,430-1,268	0,271	241
HDL-C Q1	1,231	0,690-2,195	0,482	241
HDL-C Q4	0,686	0,390-1,207	0,190	241
LDL-C T1	0,413	0,238-0,718	0,002	243
LDL-C T≥2	2,419	1,394-4,199	0,002	243
LDL-C T3	1,556	0,904-2,677	0,133	243
LDL-C Q1	0,305	0,167-0,557	<0,0001	243
LDL-C Q4	1,709	0,952-3,069	0,071	243
Triglycerides T1	0,951	0,555-1,629	0,855	241
Triglycerides T≥2	1,051	0,614-1,800	0,855	241
Triglycerides T3	1,888	1,092-3,264	0,022	241
Triglycerides Q1	0,989	0,552-1,772	0,970	241
Triglycerides Q4	1,628	0,898-2,953	0,107	241
BMI T1	0,560	0,117-0,990	0,045	221
BMI T≥2	1,785	1,010-3,155	0,045	221
BMI T3	1,438	0,823-2,512	0,201	221
Age T1	1,453	0,848-2,487	0,173	244
Age T≥2	0,688	0,402-1,179	0,173	244
Age T3	0,833	0,430-1,416	0,499	244

LDL-C: Low Density Lipoprotein; HDL-C: High Density Lipoprotein, BMI: Body Mass Index, T: tertile level, Q: quartile level; CI: Confidence Interval.

Additional Table 2: Breast Cancer Treatment

Treatment	No. of Patients	%
Surgery		
Mastectomy	64	26,2
Breast Conserving	180	73,8
Chemotherapy	165	67,6
Trastuzumab	29*	11,9
Radiotherapy	191	78,3
Endocrine Therapy	171	70,1

* 13 cases Her2 Type; 16 cases Luminal B type

Additional Table 3: Gene expression of LDL-C treated MDA MB 231

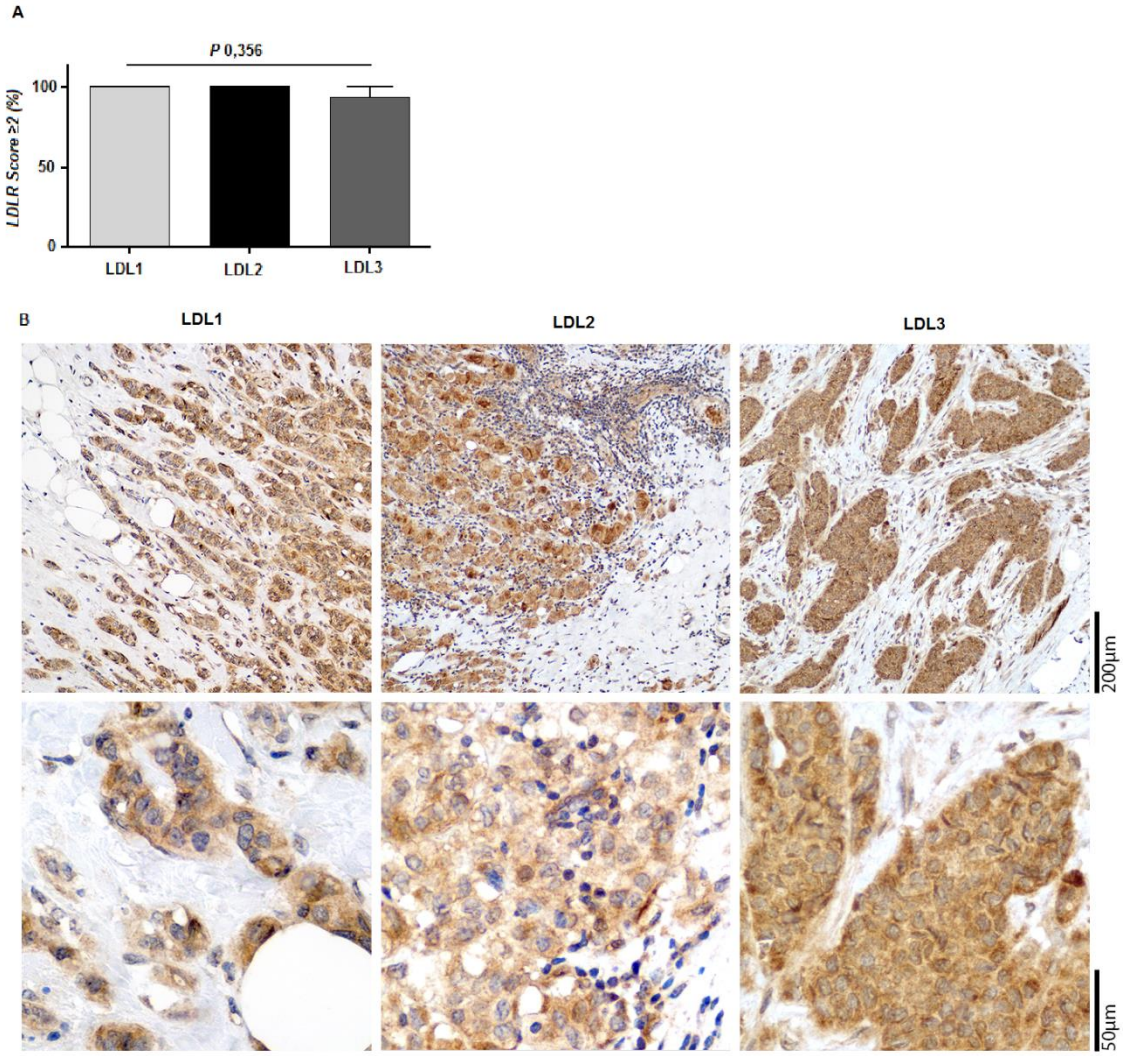
Molecules in Network	Score	Focus Molecules	Top Functions
Up regulated 6h			
ANGPTL4,FFAR3,GPR152, KIR2DL3,MAL2,MTRF1L,POLR3G, RN7SK,ROPN1,SLC25A20,SPRR2G	28	12	Organismal Development, Lipid Metabolism
CISH,CPT1A,IL24,PDGFD, PRLR,RETNLB,TNIP3	25	11	Cancer, Reproductive System Disease, Cell Morphology
TMEM229A	3	1	Embryonic Development, Organ Development, Organismal Development
LGALS9C	3	1	Digestive System Development and Function, Embryonic Development, Tissue Morphology
GRAMD1B,MAPK1	3	1	Organ Morphology, Nucleic Acid Metabolism
PCDHA8	3	1	Embryonic Development, Organ Development, Organismal Development
LCE2C	3	1	Cell Death and Survival, Cellular Assembly and Organization, Cellular Development
PCDHA13	3	1	Cell Cycle, Embryonic Development, Renal and Urological System Development and Function
GGT2	3	1	Drug Metabolism, Protein Synthesis
S100Z	2	1	Cancer, Carbohydrate Metabolism, Cardiovascular System Development and Function
XCL2	2	1	Reproductive System Disease, Cellular Movement, Cell Signaling
Down regulated 6h			
BHLHE40,BTG1,CCNG2,FASN, HMGR,IDI1,INSIG1,KRT80, LIFR,MVK	17	11	Cellular Growth and Proliferation, Cellular Development, Cell Cycle
CLDN7	2	1	Cancer, Cell Cycle, Cell Morphology
TP53INP1	2	1	Endocrine System Disorders, Gastrointestinal Disease, Inflammatory Disease
AIM2	2	1	Cell-To-Cell Signaling and Interaction, Inflammatory Response, Gastrointestinal Disease
NPY1R	2	1	Energy Production, Molecular Transport, Nucleic Acid Metabolism
Up regulated 48h			
AKR1C3,ASS1,ELF3,HIST1H4A, KRT7,KRT81,MT1E,MT1F,MT1X, RARRES3,TGFB3,TIMP3,WNT5A	15	13	Cancer, Gastrointestinal Disease, Cellular Development
ALOX5AP,C15orf48,CABLES1, CD74,CRISPLD2,EPAS1,GPR56, HLADRA,KIAA1199,PHB2, S100A2,S100A4,TACSTD2	15	13	Cancer, Gastrointestinal Disease, Dermatological Diseases and Conditions
HES1,ITGB4,LPCAT3,PBX1, PTGS1,STAT5B,TNFSF10	5	6	Cell Death and Survival, Cellular Development, Cellular Growth and Proliferation
ANGPTL2	1	1	Connective Tissue Disorders, Developmental Disorder, Skeletal and Muscular Disorders
SIX1	1	1	Cellular Development, Cellular Growth and Proliferation, Tumor Morphology
Down regulated 48h			
CSF2,DHCR7,DHCR24,EGR1, HMCR,IDI1,IL11, IL24,INSIG1,MVK,KRT15	20	11	Cellular Development, Cellular Growth and Proliferation, Lipid Metabolism

Additional Table 4: Lipid Profile in Mice Trails

	Normal Diet	Hchol Diet		Normal Diet	Hchol Diet
	Tumor			Control	
Mice model/ Weight/Lipid Profile variable	**MDA MB231 BALB SCID N=8**	**MDA MB231 BALB SCID N=8**	***P value***	**MDA MB231 BALB SCID *N=5***	**MDA MB231 BALB SCID *N=5***
Weight (g)	19,6(±1,79)	19,9(±2,87)	0,6389	20,92(±1,50)	19,9(±1,85)
TC (mg/dl)	126,8(±0,73)	372,3(±107,66)	0,0095	102,1(±17,42)	356(±84,79)
LDL (mg/dl)	11,8(±25,19)	79,8(±17,29)	0,0095	9,7(±4,41)	66(±23,29)
HDL (mg/dl)	40,3(±17,44)	106,8(±55,78)	0,0114	36,8(±8,76)	117,2(±42,23)
Triglycerides (mg/dl)	166,8(±47,77)	200(±34,33)	0,2571	271(±79,92)	114,7(±26,54)
	HTB 20 BALB SCID N=3	**HTB 20 BALB SCID N=3**	***P value***	**HTB 20 BALB SCID N=2**	**HTB 20 BALB SCID N=2**
Weight (g)	19,9 (±1,90)	19,4(±1,82)	0,700	19,6(±1,06)	17,0(±2,12)
TC (mg/dl)	-	345,5(±34,05)		125	262
LDL (mg/dl)	16,5(±14,85)	82(±3,46)	0,200	13,5(±2,12)	111(±14,14)
HDL (mg/dl)	-	120(±11,728)		-	-
Triglycerides (mg/dl)	121	123,5(±3,54)		142	-
	4T1 NOD SCID N= 4	**4T1 NOD SCID N=**	***P value***	**4T1 NOD SCID N=2**	**4T1 NOD SCID N=2**
Weight (g)	19,1(±2,85)	20,2(±2,17)	1,000	21,1(±0,40)	23,3(±0,85)
TC (mg/dl)	95,67(±22,9)	278,7(±58,11)	0,0071	199,0(±173,95)	333,5(±99,70)
LDL (mg/dl)	17,3(±4,04)	105,3(±47,16)	0,0323	5(±5,65)	50,5(±23,33)
HDL (mg/dl)	23(±7,94)	53,7(±16,92)	0,0468	48(±43,84)	54,5(±34,65)
Triglycerides (mg/dl)	93,7(±49,17)	157(±58,39)	0,2240	250(±207,89)	317,5(±135,06)

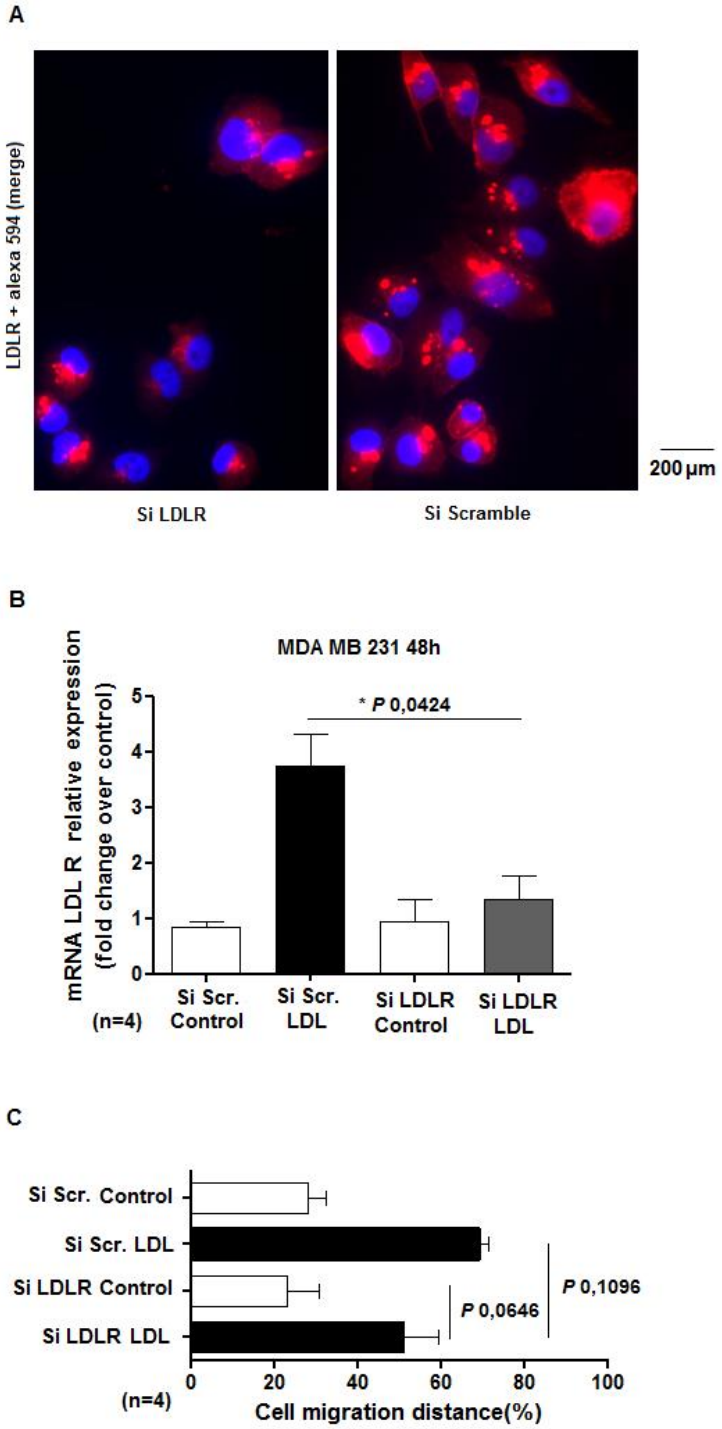
Hchol: Hypercholesterolemic ;TC: Total cholesterol; LDL: LDL-C: Low Density Lipoprotein; HDL:HDL-C: High Density Lipoprotein. Values are represented by mean±SD. *P* value : Student t test.

Additional Figures



Additional figure 1: LDLR expresion in human breast tumors

A. LDLR staining (1:100; ab30532 from Abcam, liver as positive control) was performed in breast cancer samples of 14 consecutive patients of each LDL-C tertile group of the studied cohort population (each tercile group represents groups of patients with increasing systemic LDL-C levels).
Measurements of LDLR expression was based on topographic score (0 to +3) of antibody staining tissue distribution accessed under microscopy by two independent observers. Measurements of both markers were made based on topographic score (0-+3) of antibody tissue distribution and cell intensity accessed under microscopy by two independent observers. LDLR expression was observed in primary tumors of all patients without significant variations. **B**. Representative photos of each group are shown. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ±SEM. LDL:LDL-C: Low Density Lipoprotein.



Additional figure 2: Inhibition of LDLR does not prevent the accumulation of intracellular cholesterol and LDL-C induced phenotype

If the cholesterol induced phenotype is dependent on LDL-C signaling, we asked if reduction in LDL-cholesterol up take would produce phenotype attenuation, by reducing intracellular cholesterol. To this aim we performed functional tests of proliferation and migration in cells transfected with siRNA for LDLR, a major LDL-cholesterol acceptor. We could demonstrate inhibition of LDLR expression(A), but this did not significantly reduce cell proliferation(B) and migration (B) induced by the presence of LDL-C.

Publications

Rodrigues Dos Santos C, Fonseca I, Dias S, Mendes de Almeida JC**. Plasma level of LDL-cholesterol at diagnosis is a predictor factor of breast tumor progression**. *BMC Cancer*. 2014 Feb 26;14:132 (highly accessed paper; impact factor 3.32; 1 citation). Full paper in attachment.

Santos, CR; Mendes Almeida JC and Dias, S, **Systemic LDL Promotes Breast Cancer Progression;** *Annals of Surgical Oncology*, May 2012, Volume 19 (Issue 2 Supplement);100-01 (impact factor 3,94).

Rodrigues dos Santos C, Domingues G, Matias I, Matos J, Fonseca I, de Almeida JM, Dias S **LDL-cholesterol signaling induces breast cancer proliferation and invasion** *Lipids Health Dis.* 2014 Jan 15;13(1):16. (Highly accessed paper, Article is amongst the highest ever scored in this journal (ranked #49 of 312) and in the top 25% of all articles ever tracked by Altmetric; impact factor 2,31; 2 citations). Full paper in attachment.

Presentations

“The role of the Blood Lipid Profile in Breast Cancer Progression”

American College of Surgeons Join Meeting New Jersey Chapter/Portuguese Chapter, Lisboa, April 2012

Authors: Catarina Rodrigues dos Santos, Isabel Fonseca, JC Mendes Almeida, Sérgio Dias (Invite, oral communication)

“Metabolismo Lipídico e Cancro da Mama”

XIV Congresso Anual da Associação Portuguesa de Nutrição Entérica e Parentérica, Instituto de Medicina Molecular, Lisboa, July 2012

Authors: Catarina Rodrigues dos Santos (Invite, oral communication)

“Systemic Cholesterol Promotes Breast Cancer Progression”

VIII Congresso Nacional da Sociedade Portuguesa de Senelogia, Porto, November 2012

Authors: Catarina Rodrigues dos Santos, JC Mendes Almeida, Sérgio Dias (oral communication)

"The influence of Systhemic Cholesterol in Breast Cancer Progression and Lymph Node Metastasis" Joint TuMIC- Metastasis Research Society, Champalimaud Foundation Conference, June 2012
Authors: Catarina Rodrigues dos Santos, Sérgio Dias (poster)

"Systemic Cholesterol Promotes Breast Cancer Agressiveness" 34th Annual San Antonio Breast Cancer Symposium, San Antonio, Texas, USA, December 2011
Authors: Catarina Rodrigues dos Santos, Isabel Fonseca, JC Mendes Almeida, Sérgio Dias (poster)

"Systemic Low Density Lipoprotein and Breast Cancer Progression"13th Annual Meeting of the American Society of Breast Surgeons, Phoenix, Arizona, USA, Maio 2012
Authors: Catarina Rodrigues dos Santos, Isabel Fonseca, JC Mendes Almeida, Sérgio Dias (poster)

"Cholesterol Promotes Breast Cancer Growth" A One Day Symposium sponsored by EACR, Porto, October 2012
Authors: Catarina Rodrigues dos Santos, Isabel Fonseca, JC Mendes Almeida, Sérgio Dias (poster)

Awards

First award of Sociedade Portuguesa de Senelogia (2012)

Papers