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Ciências da Saúde

Cyclooxygenase-2 Immunoexpression in Breast Cancer: Progesterone Receptor Influence

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Resumo

No cancro da mama a expressão de ciclooxigenase-2 está relacionada com elevados níveis locais de receptor de estrogénio, e conseqüente pior prognóstico, mas a relevância clínica da ciclooxigenase-2 ainda não está bem esclarecida.

Foi analisada, por imunohistoquímica, a expressão de ciclooxigenase-2 e do receptor de progesterona em 31 casos de carcinoma ductal invasivo, de doentes do Departamento da Mulher e da Criança do Centro Hospitalar Cova da Beira.

A expressão de ciclooxigenase-2 e do receptor de progesterona foi verificada em 64.5% e 54.8% dos tumores, respectivamente. Verificou-se que os tumores com expressão do receptor de progesterona tinham menores dimensões, e a maioria das mulheres com estes tumores não apresentava metastização ganglionar, quando comparados aos tumores com receptor de progesterona negativo. Foram encontrados resultados semelhantes quando se correlacionou a expressão do receptor de progesterona e de ciclooxigenase-2 com os factores clinicopatológicos.

Estes resultados sugerem que o receptor de progesterona tem uma função protectora no cancro da mama, pela modulação da via inflamatória. Os carcinomas ductais invasivos da mama que exprimem COX-2⁺/PR⁺, têm melhor comportamento biológico, e sugerimos que a determinação da ciclooxigenase-2 poderá vir a ter utilidade na prática clínica. Estudos posteriores poderão vir a clarificar o papel da determinação imunohistoquímica da ciclooxigenase-2 no cancro da mama.

Palavras-chave

Cancro da mama, Ciclooxigenase-2, Receptor de Progesterona, Receptor de Estrogénio.

Resumo Alargado

O processo inflamatório crónico está associado com o cancro da mama, as citocinas libertadas por esta via aumentam a expressão de ciclooxygenase-2. Esta enzima induz a produção de prostaglandinas, com conseqüente aumento dos níveis de aromatase e de estrogénio. Vários estudos indicam que os tumores do tipo receptor de estrogénio positivo, receptor de progesterona negativo, são os tumores mais agressivos, quando comparados com tumores com receptores de progesterona positivos. No entanto, a função do receptor de progesterona, no cancro da mama, não é clara. Neste estudo tentou-se estabelecer uma possível relação entre o receptor de progesterona e a ciclooxygenase-2, correlacionando-os com factores clinicopatológicos.

Analisou-se, por imunohistoquímica, a expressão de ciclooxygenase-2 e de receptor de progesterona, em carcinomas invasivos ductais de 31 doentes do Departamento da Mulher e da Criança do Centro Hospitalar Cova da Beira, entre os anos de 2007 e 2009. A expressão de ciclooxygenase-2 e do receptor de progesterona foram correlacionadas com factores clinicopatológicos (idade, dimensão tumoral, grau de diferenciação tumoral e metastização ganglionar).

Verificou-se, que 64.5% (n = 20) dos tumores apresentavam expressão de ciclooxygenase-2 e 45.2% (n = 14) dos tumores apresentavam expressão do receptor de progesterona. Quando estes dados foram correlacionados com os factores clinicopatológicos, foi possível estabelecer uma relação entre o receptor de progesterona, a dimensão tumoral e a metastização ganglionar. Obtiveram-se resultados semelhantes, quando a expressão do receptor de progesterona e a expressão de ciclooxygenase-2 foram correlacionados com os mesmos parâmetros. Verificou-se que tumores com expressão de receptor de progesterona tinham menores dimensões e o número de casos com metastização ganglionar era reduzido. Estes dados sugerem que o receptor de progesterona é um modelador da ciclooxygenase-2, podendo desempenhar uma função anti-inflamatória, no entanto, são necessários mais estudos, com maior número de casos clínicos, de modo a que doentes com cancro da mama possam beneficiar de uma terapêutica mais eficaz.

Palavras-chave

Cancro da mama, Ciclooxygenase-2, Receptor de Progesterona, Receptor de Estrogénio.

Abstract

In breast cancer cyclooxygenase-2 expression is related with high local estrogen receptor levels and consequent poor outcomes, but the clinical relevance of cyclooxygenase-2 is still unclear.

We analyzed, by immunostaining, cyclooxygenase-2 and progesterone receptor expression in 31 cases of women with invasive ductal carcinoma, from Child and Women department of Cova da Beira Medical Center.

Cyclooxygenase-2 and progesterone receptor expression was observed in 64.5% and 54.8% of the tumors, respectively. We verified that tumors with progesterone receptor expression had lower size and the majority of women with these tumors had no axillary node metastasis, when compared to tumors with positive progesterone receptor. Similar results were found when a correlation between progesterone receptor, cyclooxygenase-2 and clinicopathological factors was performed.

These results suggest that progesterone receptor has a protective role in breast cancer by inflammatory pathway modulation. COX-2⁺/PR⁺ seems to be a marker of better behavior in ductal invasive breast cancer.

We speculate if cyclooxygenase-2 determination may have be a clinical usefulness in clinical practice. It's expected that further studies may clarify this issue.

Keywords

Breast cancer, Cyclooxygenase-2, Progesterone Receptor, Estrogen Receptor.

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Lista de Acrónimos

A

AA	Arachidonic acid
AF-1	Activation function 1
AF-2	Activation function 2
Akt	Protein Kinase B

B

BRCA1	Breast Cancer Type 1
BRCA2	Breast Cancer Type 2

C

cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRE	cAMP response element
CTL	Cytolytic T lymphocyte
CYP19	Cytochrome protein 450, subfamily XIX

D

DBD	DNA binding domain
DC	Dendritic cells
DNA	Deoxyribonucleic acid

E

EGF	Epidermal growth factor
ER	Estrogen receptors
ER α	Estrogen receptors alpha
ER β	Estrogen receptors beta
ERK	Extracellular signal-regulated protein kinase

H

HER-2	Human epidermal growth factor receptor 2
HIF1- α	Hypoxia inducible factor 1 alpha
HRP	Horseradish peroxidase
HSPG	Prostaglandin H2 synthase

I

IL-1	Interleukine 1
IL-1B	Interleukine 1beta
IL-4	Interleukine 4
IL-6	Interleukine 6
IL-10	Interleukine 10
IL-12	Interleukine 12
IL-23	Interleukine 23
IκB	Kappa b inhibitor
IFN-γ	Interferon-gamma

J

JNK	c-Jun N-terminal kinase
-----	-------------------------

L

LBD	Ligand binding domain
LPS	Lipopolysaccharides

M

MAPK	Mitogen-activated protein kinase
MEKK1	MAPK kinase 1

N

NSAID's	Non-steroidal anti-inflammatory drugs
NF-IL6	Nuclear factor interleukin 6
NF-κB	Nuclear factor kappa B
NK	Natural killer cells

P

PDGF	Growth factor derived from platelets
PG	Prostaglandins
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2	Prostaglandin F2
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGI2	Prostaglandin I2
PI3K	Phosphoinositide 3-kinase

PKC- ζ	Protein kinase C-zeta
PR	Progesterone receptor
PR-A	Progesterone receptor-A
PR-B	Progesterone recepto-B
PR-C	Progesterone receptor-C

R

ROS	Reactive oxygen species
Raf-1	Murine leukemia viral oncogene homolog 1
Ras	Rat sarcoma viral oncogene

S

Scr	Serum creatinine response
STAT3	Signal transducer and activator of transcription 3

T

Th1	T helper type 1
TNF- α	Tumor necrosis factor-alpha
TP53	Tumor Protein 53
TRAIL	Apoptosis-inducing ligand related to the tumor necrosis factor
TXA2	Thromboxane A2

V

VEGF	Vascular endothelial growth factor
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Chapter 1: Introduction

Cancer is a worldwide disease, it was estimated that in Europe in 2008 there were 3.2 million new cancer cases and 1.7 million deaths from cancer, and breast cancer accounted for 420.850 new cases, leading to death 129.390 women (Ferlay et al. 2010). In the same year, in Portugal, it is estimated that there were 5.280 new cases and 1.520 deaths from breast cancer (Ferlay et al. 2010). This represents a matter of great concern because death by breast cancer is the most important cause of cancer mortality in women between 40 and 50 years old (Danaei et al. 2005).

Despite being a disease more common in westernized countries, there is some variability in incidence and mortality in these countries, probably associated to different behavioral and environmental risk factors (Danaei et al. 2005; Ruddon 2007).

The etiology of breast cancer is unknown, but inflammation plays an important role in breast cancer development. The mechanisms involving the inflammatory process in breast cancer genesis are poorly understood (Lu et al. 2006).

Inflammation and Carcinogenesis

Carcinogenic Risk Factors

The most important factors associated with carcinogenic risk for breast cancer, are heredity, persistent hormonal stimulation and chronic inflammation (Henderson and Feigelson 2000; Ruddon 2007).

About 10% of the breast cancers are hereditary in origin, most of them associated with a mutation in the breast cancer type 1 (BRCA1), breast cancer type 2 (BRCA2), and tumor protein 53 (TP53) genes; therefore family history is a determinant risk factor (Polyac 2007; Schlehe and Schmutzler 2008).

Hormonal factors also stimulate breast tumor growth. Hormones are considered risk factors for breast cancer, due to the promoting effect of estrogen and progesterone in carcinogenesis (Pitot and Dragan 1991). Clinical factors related with hormonal stimulation, such as early menarche, late menopause, postmenopausal obesity, hormone replacement therapy and oral contraceptives, have been implicated as epidemiologic factors associated with breast cancer (Henderson and Feigelson 2000). In those women with history of early menarche and late menopause there are a lot of ovulatory cycles, with long and intensive exposure to estrogen

and progesterone (Henderson and Feigelson 2000). In adipose tissue androgens, by aromatase enzymatic action, are converted into estrogen (Henderson and Feigelson 2000; Ruddon 2007). Postmenopausal obese women have increased free estrogen levels in circulation (Henderson and Feigelson 2000; Ruddon 2007). Women in hormone replacement therapy, have increased mammographic breast density, consequence of estrogenic breast stimulation, which seems to be a risk factor for breast cancer (Ross et al. 2000). In the Ross et al. study 2,500 postmenopausal women in hormone replacement therapy for every 5 years of use, the risk of breast cancer increased 10% (Ross et al. 2000). The risk of oral contraceptives for breast cancer development is more controversial, however it should be considered when used before age of 20 (Henderson and Feigelson 2000; Ruddon 2007). Probably long and continued expositions to pill estrogen and progestin have a promoting effect on breast carcinogenesis (Henderson and Feigelson 2000). Hormonal factors have been considered as risk factors for breast cancer, due to the promoting effect of estrogen and progesterone in carcinogenesis (Pitot and Dragan 1991).

It is estimated that the pathogenesis of 15-20% human tumors is related to chronic inflammation (Karin and Greten 2005; Allavena et al. 2008). Chronic inflammation has been considered as a risk factor for breast cancer, probably, in consequence of continuous release of inflammatory mediators that impair the tissue regeneration and enhance tumor growth (Hu et al. 2008).

Inflammatory Pathway

Inflammation is a physiological process in response to foreign agents from self (Lu et al. 2006). The first line of defense of the inflammatory response is the innate immune response at this stage there is migration of neutrophils, macrophages, mast cells, dendritic cells (DC) and natural killer cells (NK) to inflammation site (Lu et al. 2006; DeNardo and Coussens 2007). These cells have the ability to release inflammatory mediators such as interleukin 10, 12 and 23 (IL-10, IL-12, IL-23) and tumor necrosis factor-alpha (TNF- α), which may enhance the capacity of phagocytosis, neutralization or elimination of the foreign agent (DeNardo and Coussens 2007; Lin and Karin 2007). Almost simultaneously to the innate immune response, adaptive immune response occurs, where the DC present antigens to T cells, leading them into inflammation site, followed by B cells, with subsequent clonal expansion and destruction or neutralization of the antigen (DeNardo and Coussens 2007). When foreign agents are completely neutralized or destroyed, the inflammatory process ceases, however, in the event of deregulation of acute inflammatory process, sets up a chronic inflammation (Lu et al. 2006; DeNardo and Coussens 2007).

Rudolf Virchow, in nineteenth century, was the first to suggest a link between chronic inflammation and cancer, noting that some irritants, when they were not eliminated, cause

chronic inflammation and cell proliferation, and increase cancer risk (DeNardo and Coussens 2007). Currently, epidemiological studies refer that inflammation associated with cancer accounts for 15-20% of worldwide deaths, and this relationship is due to two routes, intrinsic and extrinsic pathways (Mantovani et al. 2008).

The intrinsic pathway is activated by genetic events such as activation of oncogenes by mutation, chromosomal rearrangement or amplification and inactivation of tumor suppressor genes events that promote cellular alterations with subsequent release of inflammatory mediators (Mantovani et al. 2008). The extrinsic pathway is triggered by infectious or inflammatory events that increase the risk of developing cancer in certain anatomic sites (Mantovani et al. 2008).

The activation mechanisms of the two pathways are different, however, converge on activation of transcription factors like the nuclear factor kappa B (NF- κ B), signal transducer and activator of transcription 3 (STAT3) and hypoxia inducible factor 1 alpha (HIF1- α) (Mantovani et al. 2008). These factors recruit and activate inflammatory cells and coordinate the production of inflammatory mediators such as cytokines, which activate transcription factors in inflammatory cells (Mantovani et al. 2008). Production of inflammatory mediators by transcription factors, results in an inflammatory micro-environment (see figure 1), characterized by tissue damage and malignant growth (Lu et al. 2006; Mantovani et al. 2008).

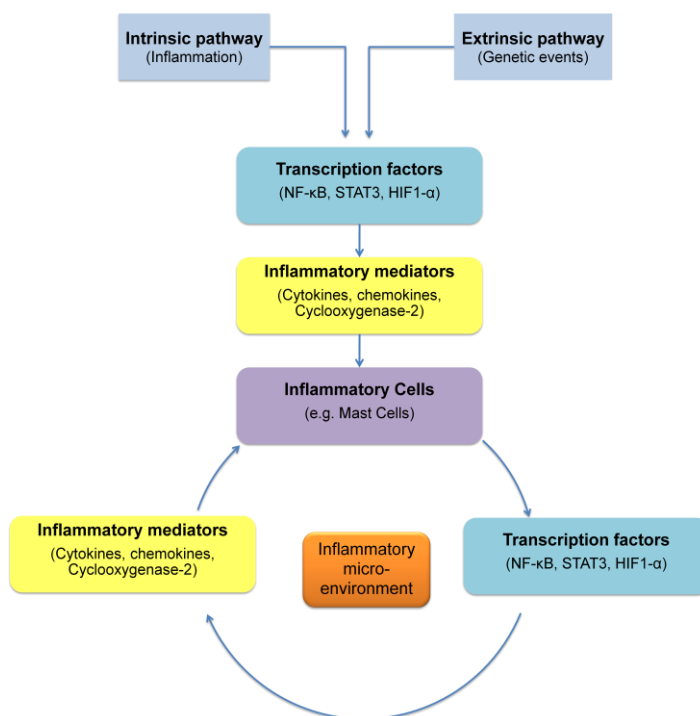


Figure 1: Intrinsic pathway is activated by genetic events, whereas extrinsic pathway is activated by inflammatory or infectious events, both culminate on NF- κ B activation, and other transcription factors, that recruit and active inflammatory cells through inflammation mediators as cytokines, more transcription factors are activated, resulting in an inflammatory micro-environment.

The microenvironment of chronic inflammation is dominated by macrophages, which are divided into two classes M1 and M2, having, respectively, anti-inflammatory or pro-tumorigenic properties and may be involved in carcinogenesis, tumor invasion and metastasis (Karin and Greten 2005; Lu et al. 2006; Mantovani et al. 2008).

M1 class when exposed to interferon-gamma (IFN- γ) results in IL-12, IL-23 and IFN- γ synthesis (see figure 2) (Karin and Greten 2005).

IL-12 confers host resistance to tumor, promoting T helper type 1 (Th1) adaptive immunity, cytolytic T lymphocyte (CTL) response and induction of IFN- γ (Lin and Karin 2007; Grivennikov et al. 2010).

IL-23 belongs to the IL-12 super-family, however, may have an anti or pro-tumorigenic effect (Lin and Karin 2007). The anti-tumorigenic effect increased proliferation of memory T cells, IFN- γ and IL-12 (Lin and Karin 2007).

IFN- γ has a toxic effect on cancer cells and angiogenic activity, induces apoptosis-inducing ligand related to the tumor necrosis factor (TRAIL) production by monocytes, NK and DC cells (Karin and Greten 2005; Lin and Karin 2007).

TRAIL induces apoptosis in various tumor cells, however, requires the inhibition of NF- κ B or neutralization of the activator of NF- κ B (TNF- α), if this inhibition does not occur, the tumors become resistant to the TRAIL cytotoxicity (Lin and Karin 2007).

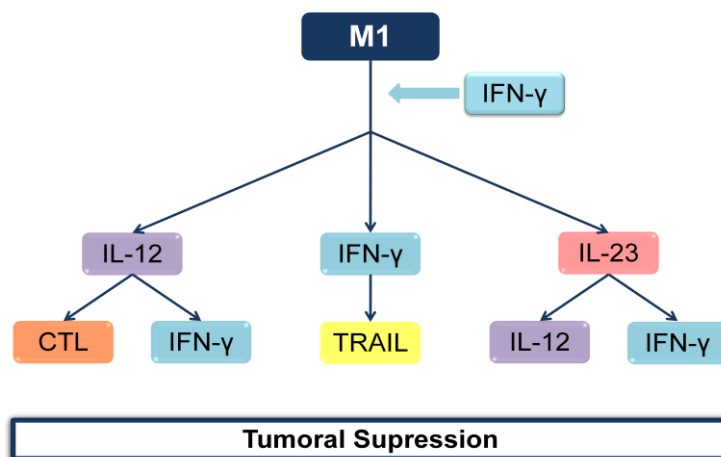


Figure 2: M1 class when exposed to IFN- γ induces IL-12, IL-23 and IFN- γ , which recruit more inflammatory mediators that participate in tumor suppression, by events such as phagocytosis and apoptosis.

Class M2 when exposed to interleukine 4 (IL-4) originates interleukine 1 (IL-1), interleukine 6 (IL-6), IL-10 and TNF- α formation (see figure 3) (Balkwill et al. 2005; Karin and Greten 2005).

Macrophages produce IL-1 that is abundant in tumorigenic sites (Lin and Karin 2007; Allavena et al. 2008). This cytokine expressed constitutively NF- κ B, which up-regulates cyclooxygenase-2 (COX-2) expression and induces HIF1 α expression, resulting in vascular endothelial growth factor (VEGF) production by stromal cells, increasing invasive and metastatic ability of tumor cells (Balkwill et al. 2005; Karin and Greten 2005; Lu et al. 2006).

IL-6 is also an inflammatory mediator and an anti-apoptotic factor and is considered a key factor in tumor growth (Lin and Karin 2007; Allavena et al. 2008). IL-6 levels increases with increasing age due to loss of inhibitory sex hormones, resulting in inhibition of DC maturation; cytokine signal transduction of this cytokine is performed by STAT3 (Balkwill et al. 2005; Lin and Karin 2007).

IL-10 is a cytokine with pro and anti-tumorigenic effects (Lin and Karin 2007). Its anti-tumorigenic effect is TNF- α and IL-6 inhibition, inhibiting NF- κ B activation, having anti-angiogenic properties (Lin and Karin 2007). However, his pro-tumorigenic effect occurs by STAT3 activation, blocking DC maturation, repression of M1 polarization and suppression of cytotoxic activity of macrophages (DeNardo and Coussens 2007; Lin and Karin 2007).

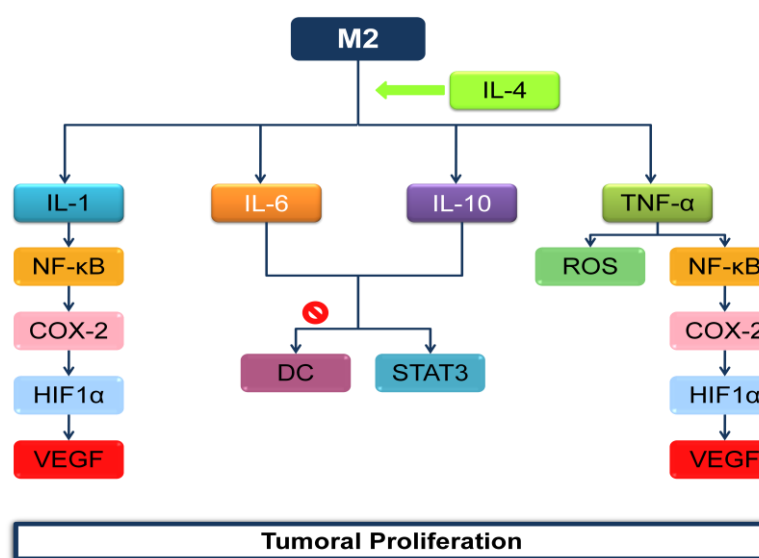


Figure 3: M2 class when exposed to IL-4 induces IL-1, IL-6, IL-10 and TNF- α , which recruits more inflammatory mediators and transcription factors. COX-2 is expressed as a result of this mast cells class and together with other inflammatory mediators confers tumor proliferation, inhibiting apoptosis and stimulating angiogenesis.

TNF- α is a pro-inflammatory cytokine, eradicates anti-tumor immunity and accelerates progression, angiogenesis and metastasis of various cancers (Lin and Karin 2007; Schetter et al. 2010). TNF- α activates NF- κ B, which by COX-2 induction activates HIF1 α , which induces angiogenic factors, contributing for proliferation and cancer cells survival (Karin and Greten 2005; Lu et al. 2006; Allavena et al. 2008; Schetter et al. 2010), and produces reactive

oxygen species (ROS) which cause deoxyribonucleic acid (DNA) damage and inhibit DNA repair (Lu et al. 2006; Lin and Karin 2007).

Tumor development depends on the collective balance between cytokines, which can be pro or anti-inflammatory (Schetter et al. 2010). When anti-tumor activity overlaps immunosuppressive activity, there is activation of transcription factors that enhance invasion and metastasis of tumor cells (Lin and Karin 2007).

One of the transcription factors that potentiate tumor development is the HIF1- α , which promotes chronic inflammation, tumor growth and angiogenesis via the transcription of VEGF (Lu et al. 2006).

STAT3 promotes inflammatory microenvironment development, proliferation, apoptosis resistance and immune tolerance (Lu et al. 2006; Grivennikov et al. 2010). It is constitutively activated in inflammatory cells and tumor cells by pro-inflammatory cytokines, lying in different types of cancer (Lu et al. 2006).

NF- κ B regulates several inflammatory molecules, phagocytosis and suppresses apoptosis leading to tumor growth and proliferation (Lu et al. 2006). NF- κ B is considered the key factor between inflammation, promotion and tumor progression, because it becomes active after an inflammatory stimulus (Karin and Greten 2005; Schetter et al. 2010). This transcription factor acts binding to kappa B inhibitor (I κ B) that is found in the cytoplasm, the I κ B is phosphorylated and enters in proteasomal degradation after an inflammatory stimulus, NF- κ B is released and enters in the nucleus where it activates COX-2 transcription, which is over-expressed in various types of cancer (Schetter et al. 2010).

Cyclooxygenase

The cyclooxygenase (COX) or prostaglandin H₂ synthase (HSPG) is an enzyme that catalyzes the prostaglandins (PG) production from arachidonic acid (AA) (Singh-Ranger et al. 2008). Exists in two isoforms, cyclooxygenase-1 (COX-1) and COX-2, the first is constitutively expressed in different tissues including platelets, gastric mucosa, kidney, being responsible for maintaining homeostasis in these tissues (Isakson 2003; Schetter et al. 2010). The second isoform was discovered in 1992, is expressed in most tissues, is the induced form in response to growth factors, tumor promoters, hormones and cytokine (Isakson 2003; Singh-Ranger et al. 2008).

The COX-2 expression and subsequent increase of transcription factors such as HIF1- α and prostaglandins play a key role in mediating the inflammatory response, may affect cell

proliferation, DNA mutation rates, angiogenesis and metastasis, and is a potential therapeutic target in treating breast cancer (Isakson 2003; Singh-Ranger et al. 2008; Schetter et al. 2010).

COX can be inhibited by non-steroidal anti-inflammatory drugs (NSAID's) or by selective inhibition of COX-2 such as celecoxib (Schetter et al. 2010). Continued use of these drugs has been associated with reduction of certain cancers such as breast cancer, confirming the relationship between COX and the inflammatory process; however, these drugs may cause adverse effects such as renal and cardiovascular toxicity (Schetter et al. 2010).

Cyclooxygenase-2 Up-regulation Mechanisms

There is evidence that several mechanisms contribute to the COX-2 overexpression in tumor tissues (Howe et al. 2001).

COX-2 human promoter contains multiple binding sites for transcription factors that respond to different stimuli such as cyclic adenosine monophosphate (cAMP), cAMP response element (CRE), nuclear factor interleukin 6 (NF-IL6) and NF- κ B (Howe et al. 2001).

These factors induce COX-2 expression in response to stimuli like lipopolysaccharides (LPS), growth factor derived from platelets (PDGF), interleukin 1 beta (IL-1 β) and TNF- α (see figure 4) (Howe et al. 2001; Davies et al. 2002; Dannenberg and Howe 2003; Bassères and Baldwin 2006).

PDGF induces COX-2 by activation of rat sarcoma viral oncogene/murine leukemia viral oncogene homolog 1/extracellular signal-regulated protein kinase (Ras/Raf-1/ERK) and rat sarcoma viral oncogene/mitogen-activated protein kinase kinase 1/c-Jun N-terminal kinase (Ras/MEKK1/JNK) signal transduction pathways and is predominantly mediated by the CRE (Howe et al. 2001).

The inflammatory mediators IL-1 β and TNF- α degrade I κ B via phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), NF- κ B is released and induces COX-2 transcription (Bassères and Baldwin 2006).

COX-2 induction by LPS occurs via mitogen-activated protein kinase (MAPK) and via protein kinase C-zeta (PKC- ζ) (Davies et al. 2002). MAPK can also be activated by ceramide, which activates JNK; leading to COX-2 and AA increased expression, which results in PG increased production (Howe et al. 2001; Davies et al. 2002).

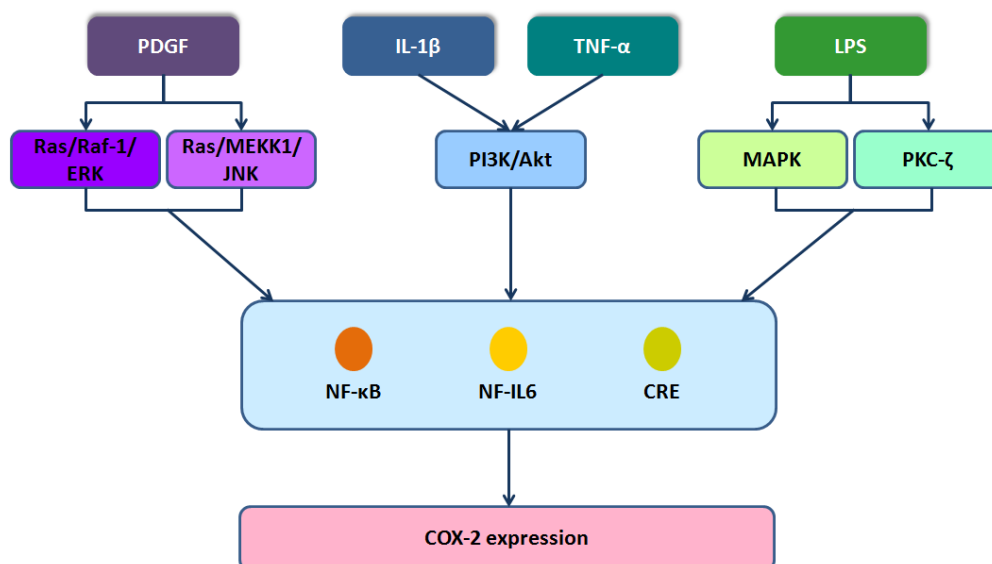


Figure 4: COX-2 is up-regulated by PDGF that induces COX-2 via Ras/Raf-1/ERK and Ras/MEKK1/JNK, by IL-1 β and TNF- α via PI3K/Akt and NF- κ B and by LPS via MAPK and PKC- ζ , these signal transduction pathways bind to COX-2 human promoter, inducing its over-expression.

Prostaglandins Synthesis

AA released from phospholipids membrane by phospholipase A2 action is metabolized by COX action (Singh-Ranger et al. 2008). This process occurs in two steps, in first COX inserts molecular oxygen in AA, prostaglandin G2 is produced (PGG2) and is converted in prostaglandin H2 (PGH2) by COX peroxidase activity (Howe et al. 2001). PGH2 by specific prostanoid isomerases action is converted in prostaglandin E2, F2, D2, I2 (PGE2, PGF2, PGD2, PGI2) and thromboxane A2 (TXA2) (see figure 5) (Howe et al. 2001; Gately and Kerbel 2003).

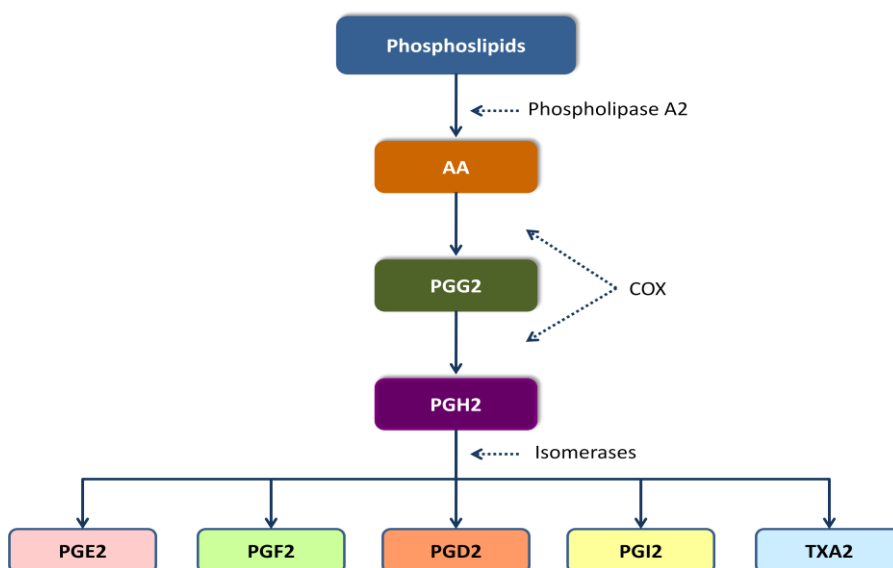


Figure 5: Phospholipase A2 synthesizes AA from phospholipids. AA is converted in PGG2 through COX-2, in turn PGG2 is converted in PG by specific isomerases.

Prostaglandins presence and levels, especially PGE₂, in human tumor tissues have been associated with the aromatase and human epidermal growth factor receptor 2 (HER-2) expression with consequent poor prognosis (Davies et al. 2002; Mendelson and Hardy 2006).

Epidermal Growth Factor Receptor 2 Synthesis

HER-2 is a transmembrane receptor protein that belongs to epidermal growth factor receptor family with over-expression occurring in approximately 30% of breast tumors (Benoit et al. 2004).

HER-2 induces NF- κ B and activates COX-2 synthesis via Ras/MAPK, resulting in prostaglandins synthesis, primarily PGE₂ (Benoit et al. 2004; Bassères and Baldwin 2006).

Benoit et al. found that COX-2 expression is related to HER-2 expression via PGE₂, and PGE₂ inhibition by celecoxib resulted in HER-2 reduced expression (see figure 6) in breast tumors (Benoit et al. 2004).

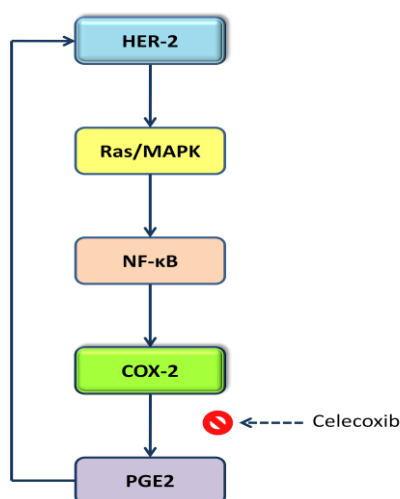


Figure 6: HER-2 induces COX-2 up-regulation via Ras/MAPK and NF- κ B, COX-2 is over-expressed and converts AA in PG. Synthesized PGE₂ stimulates HER-2, suggesting a positive feedback between COX-2 and HER-2. Use of Celecoxib (COX-2 inhibitor) has proof this theory, because it inhibits PGE₂ synthesis and as result HER-2 levels are diminished.

COX-2 increased expression by HER-2, establishes a relationship between HER-2 and aromatase, both present in breast tumors with high proliferation rate (Mendelson and Hardy 2006; Subbaramaiah et al. 2006).

Aromatase Synthesis

PGE2 stimulates cAMP signaling cascade, PKA and CRE that increase cytochrome protein 450, subfamily XIX (CYP19) gene transcription, with consequent aromatase activation (see figure 7) (Mendelson and Hardy 2006; Subbaramaiah et al. 2006). This enzyme is responsible for estrogen synthesis, Subbaramaiah et al. concluded that estrogen synthesis via the PGE2 is a consequence of COX-2 induction by the HER-2 (Mendelson and Hardy 2006; Subbaramaiah et al. 2006).



Figure 7: PGE2 induces estrogen synthesis. PGE2 via cAMP, PKA and CRE activates aromatase resulting in estrogen synthesis.

Thomas et al. found that synthesized estrogen also had positive feedback on COX-2 expression (Thomas et al. 2008). Estrogen binds to its receptor and undergoes translocation to the nucleus; this interaction activates serum creatinine response (Scr), which stimulates metalloproteins cascade, allowing epidermal growth factor (EGF) release from heparin and its binding to HER-2, which activates ERK (Thomas et al. 2008). Phospholipase A2 is a ERK substrate and its active form releases phospholipids from cell membranes, AA is formed, with consequent synthesis of PGE2 (see figure 8) (Thomas et al. 2008).

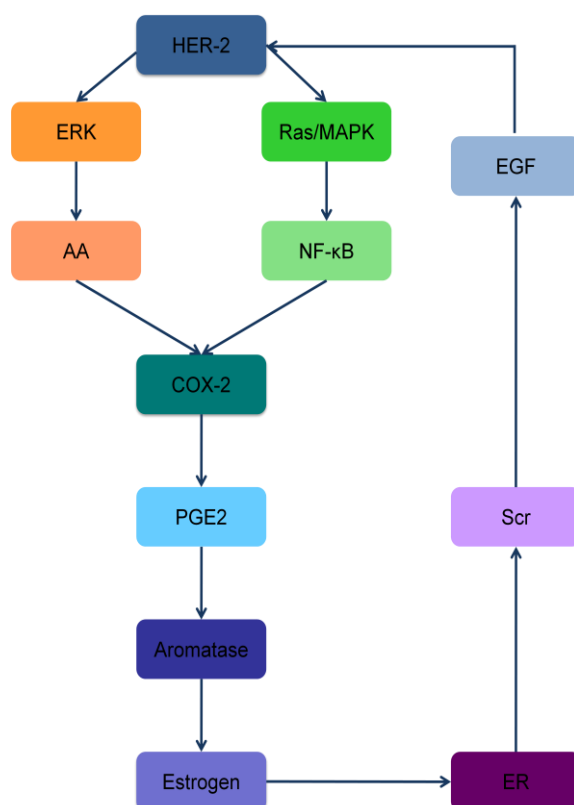


Figure 8: COX-2 synthesizes PGE2 which via cAMP induces CYP19 expression, resulting in estrogen synthesis by aromatase. Estrogen acts through its receptor and undergoes translocation to the nucleus where activates Scr, allowing EGF binding to its receptor. HER-2 acts through NF-κB activating COX-2 expression, and through ERK resulting in AA formation. More PGE2 is produced and as consequence more estrogen is synthesized by aromatase, suggesting a feedback between estrogen and HER-2.

Estrogen binding to its receptor increases inflammatory process and is involved in pathophysiology of various cancers, including breast cancer (Mendelson and Hardy 2006; Subbaramaiah et al. 2006).

Steroid Hormone Receptors

Approximately 75% of breast cancers express estrogen receptors (ER) (Cui et al. 2005). ER is a nuclear transcription regulator that can bind to transcription factors, proteins and estrogen (Cui et al. 2005; Mendelson and Hardy 2006).

There are two isoforms of this receptor that are expressed in mammary gland, estrogen receptors alpha and beta (ER α and ER β), ER α over-expression is critical to breast cancer development (Cui et al. 2005).

It has long been thought that ER induced progesterone receptor (PR) and that serves as an indicator of ER functional capacity; new studies indicate that PR is an independent predictor risk factor and have protective actions which antagonize inflammatory response pathway (Mendelson and Hardy 2006).

PR exists in three isoforms, progesterone receptor-A (PR-A) (94kDa), progesterone receptor-B (PR-B) (110kDa) and progesterone receptor-C (PR-C) (60kDa) (Abdel-Hafiz et al. 2009). PR are proteins with several domains, a DNA binding domain (DBD), N-terminally that activates proximal activation function 1 (AF-1) common to PR-A and PR-B, and a C-terminally that upstream ligand binding domain (LBD) has a nuclear localization region with activation function 2 (AF-2) (Mendelson and Hardy 2006; Abdel-Hafiz et al. 2009).

PR-A and PR-B are equimolar in most tissues, but in breast cancer this balance does not exist, PR-A can block all three PR-B transcriptional activation domains, acting as PR repressor in breast cancer (Mendelson and Hardy 2006).

PR-C isoform is augmented in cancerous breast cells, binds to PR-B reducing its capacity to interact with progesterone response elements in progesterone-responsive genes (Mendelson and Hardy 2006).

Mendelson et al. concludes that NF- κ B increased activation, results in PR-C over-expression that together with PR-A and PR-B ablation results in COX-2, HER-2 and aromatase over-expression, suggesting a protective role of RP in breast cells (Mendelson and Hardy 2006).

Progesterone Receptor and Breast Cells

In breast cells PR directly interacts with NF- κ B to block its binding to DNA, or inhibits NF- κ B activation and translocation to the nucleus via I κ B α (Mendelson and Hardy 2006). NF- κ B has a major role in COX-2 over-expression, which in turn up-regulate expression of the genes encoding aromatase and HER-2 via PGE₂, resulting in estrogen up-regulation (Mendelson and Hardy 2006).

PR impairs NF- κ B transactivation of COX-2 via induction of I κ B α , these findings suggest that NF- κ B inhibition by PR, results in estrogen lower levels and PR serves a crucial role in blocking breast tumor formation and progression (see figure 9) (Mendelson and Hardy 2006; Hardy et al. 2008).

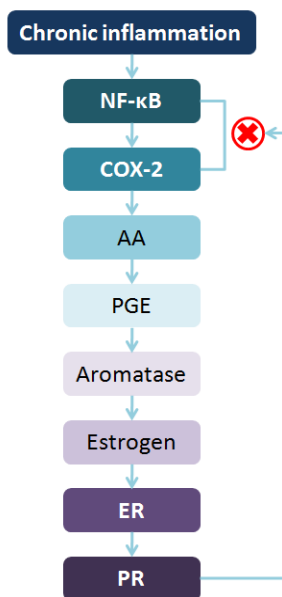


Figure 9: COX-2 is expressed in chronic inflammation, converting AA into PGE, leading to aromatase increase with subsequent increase of ER and PR. PR through NF-κB inhibition results in COX-2 lower expression.

Another suggestion of PR protective role is ER⁺/PR⁻ receptor tumor subtype that can amplify HER-2 expression, which results in a worse overall survival (Cui et al. 2005). There are four receptor tumor subtypes: ER⁺/PR⁺, ER⁺/PR⁻, ER⁻/PR⁺ and ER⁻/PR⁻, that differs with age, hormone use and menopause (Cui et al. 2005). ER⁺/PR⁻ is the most aggressive receptor tumor subtype, it has high proliferation rates and has higher recurrence than ER⁺/PR⁺, this findings also suggests that PR has a protective role in breast cancer (Arpino et al. 2005).

Based on these studies, which indicate that PR might have a protective role in breast cancer, we speculate that tumors with positive COX-2 expression and positive PR expression had an overall better prognosis than tumors with positive COX-2 expression and negative PR expression. It was performed an immunohistochemical study to determine a possible relation between COX-2 and PR expression, correlating them with clinicopathologic factors (age, tumor size, histologic grade and axillary node metastasis), which are known to be classical prognostic factors in breast cancer.

Chapter 2: Materials and Methods

Patients Selection

Child and Women department of Cova da Beira Medical Center has initiated its Gynecological Oncology activity in 2005 and since then has given assistance to several women with breast cancer.

In 2007 the department had a restructuration and started to work with the current anatomic pathology laboratory, for these reason only clinical cases from 2007 to 2009 were selected. For immunohistochemical study, it was selected 31 clinical cases that meet the follow criteria:

1. Histological invasive ductal carcinoma confirmed by an independent pathologist
2. Tumor with 10 mm or more in longest diameter (in order to material preservation for further studies)
3. Submitted to radical surgery with axillary assessment
4. No pregnant woman
5. No primary chemotherapy or hormonotherapy
6. No documented distant metastasis

Immunostaining

Sample Pre-treatment

It was selected representative pathologic material from paraffin blocks. From each selected paraffin block were obtained three 3µm sections: one slide was for hematoxylin-eosin staining and the remaining two slides were for COX-2 and PR immunostaining.

Cyclooxygenase-2 and Progesterone Receptor Immunostaining

In histological sections for immunostaining, a dewaxing was performed during 10 minutes with xylene, followed by rehydration in decreasing ethanol grades (absolute, 95% and 70%) and water. To proceed with the antigen retrieval, slides were put into pressure cooker and citrate buffer 0.01 M for 6 minutes at pH 6 and washed with water and buffer solution (K5006, ChemMat™, Dako).

The slides were then incubated with the primary antibody, for COX-2 staining was used clone SP21, rabbit monoclonal antibody (MC-16-240r, CellMarque™) at 1:10 dilution; for PR staining were used clone 16 and clone SAN27 (NCL-L-PGR-AB, Novocastra™) at 1:100 dilution, in both

cases the diluent used was S2022 (Dako Real) and incubation with the primary anti-body was made for 25 minutes at room temperature, then washed with buffer solution (K5006, ChemMat™, Dako).

Incubation with biotinylated secondary antibody was made for 25 minutes, using the bottle A from K5001 kit (Dako Real™), followed by washing with buffer solution (K5006, ChemMat™, Dako). The next step was to block endogenous peroxidase using blocking-Peroxidase solution (S2023, Dako Real™) for 7 minutes and 30 seconds, followed by washing with buffer solution (K5006, ChemMat™, Dako).

After these procedures, it was added streptavidin conjugated horseradish peroxidase (HRP) (bottle B, K5001, Dako Real), for 25 minutes followed by washing with buffer solution (K5006, ChemMat™, Dako), chromogen was added (Dako Real DAB + chromogen, bottle C + HRP substrate buffer bottle D, K5001, Dako Real) at a 20:1000 dilution for 15 minutes with washes at every 5 minutes, this was followed by washing with a buffer solution (K5006, ChemMat™, Dako). The counterstaining was done with Mayer's hematoxylin for 1 minutes and washed with a buffer solution (K5006, ChemMat™, Dako), dehydrated in increasing ethanol grades (75%, 90%, absolute) and xylene. Slides were preserved in synthetic mounting medium, and were analyzed with an optical microscope (x400), using 50 fields for slide.

For COX-2 was considered cytoplasmatic immunostaining and to PR was recorded nuclear immunostaining.

The intensity of immunostaining was recorded as: negative (-), moderate (+) or high (++) .

Positivity of immunostaining was recorded as percentage of the cells that stained. It was counted all cells in an optical high power field considered representative of all slide, in a minimum of 500 cells. For statically analysis we considered expression in less than 25% of the cell as a negative result; expression from 25 to 50% as a light expression; from 50 to 75% as moderate expression and 75 to 100% as a high expression. All cases with positive expression had a moderate or high intensity.

For comparative statistical analysis we considered as positive immunostaining cases with light, moderate or high expression.

Statistical Analysis

Was performed a comparative study between patients clinical factors (age, tumor size, differentiation grade and ganglion metastasis) and COX-2 and PR expression.

Statistical analysis was performed using SPSS, version 17. Mann-Whitney and Chi-Squared tests were used for the analysis, considering a statistical significance when P-value was < 0.05 .

Chapter 3: Results Analysis

Descriptive Statistical Analysis

The analyzed clinical cases were between the year 2007 and 2009 and the age of patients varied from 39 to 91 years old. Most of the tumors expressed COX-2 (64.52%) and 45.16% of the tumors expressed PR. It was also taking into account the differentiation grade and axillary node metastasis, and we verified that twenty four of tumors had a high (G2 or G3) differentiation grade and only 29.03% of women had axillary node metastasis (see table 1).

Table 1: Patient characteristics: Year, Age, COX-2 expression, PR expression, Tumor Size, Differentiation Grade and Axillary Node Metastasis.

Characteristics	N.º of Patients	(%)
Year:		
2007	13	41.94%
2008	15	48.39%
2009	6	19.35%
Age:		
< 40	2	6.45%
≥40 <50	2	6.45%
≥50 <60	3	9.68%
≥60 <70	6	19.35%
≥70 <80	9	29.03%
≥80 <90	8	25.81%
≥90	1	3.23%
COX-2 staining:		
Negative	11	35.48%
Moderate	18	58.06%
High	2	6.45%
COX-2 expression:		
0-25%	11	35.48%
25%-50%	14	45.16%
50%-75%	5	16.13%
75%-100%	1	3.23%
PR staining:		
Negative	17	54.84%
Moderate	10	32.26%
High	4	12.90%
PR expression:		
0-25%	17	54.84%
25%-50%	5	16.13%
50%-75%	6	19.35%
75%-100%	3	9.68%
Tumor size (mm):		
< 20	10	32.26%
≥20 <50	15	48.39%
≥50	6	19.35%
Differentiation grade:		
G1	7	22.58%
G2	16	51.61%
G3	8	25.81%
Axillary node metastasis:		
Absent	22	70.97%
Present	9	29.03%

Immunohistochemistry Analysis

Cyclooxygenase-2 Immunoreactivity

COX-2 expression was considered positive by semiquantitative scoring in 20 of 31 cases studied (64.52%), all 20 cases had at least 50% or more of COX-2 expression and all cases had a moderate or strong staining intensity. Immunostaining intensity was considered negative in 11 cases, moderate in 18 cases and strong in 2 cases (see annex 1). In figure 10 it can be seen a moderate immunostaining with moderate expression.

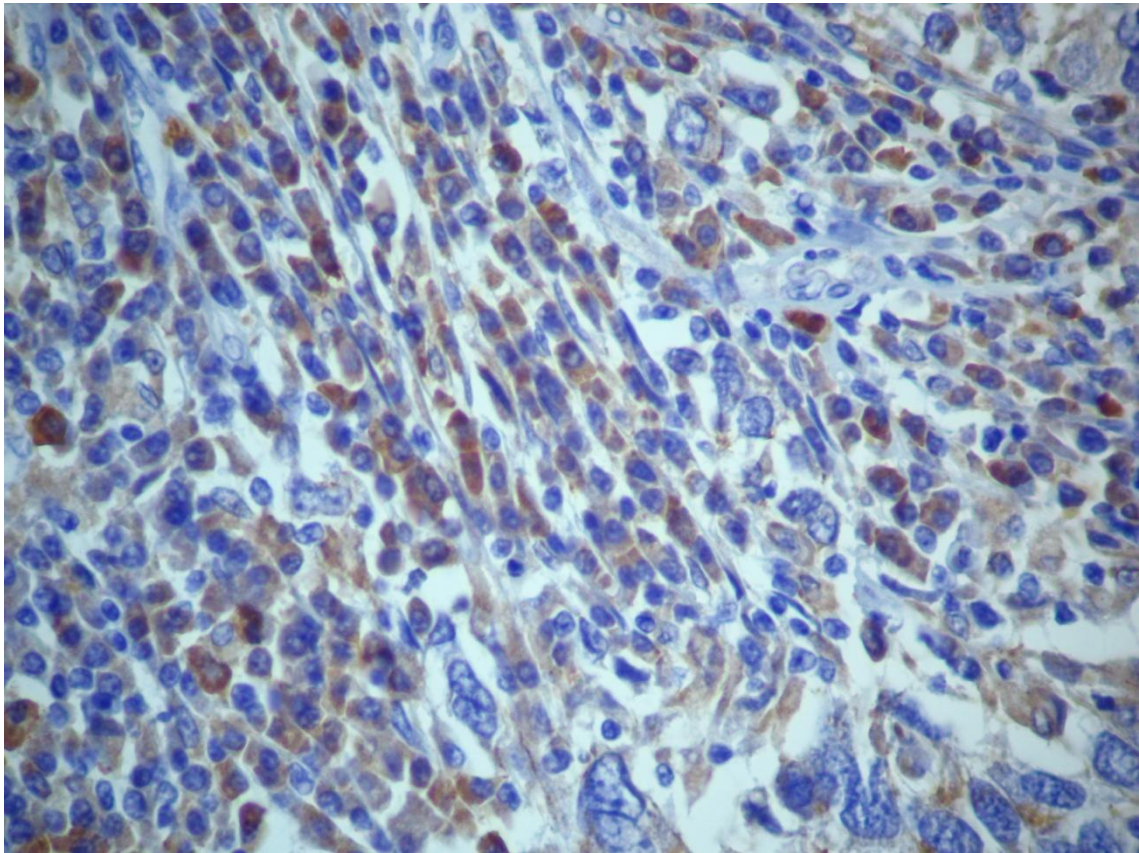


Figure 10: Immunohistochemical localization of COX-2 in ductal carcinoma (400 x magnifications). There is cytoplasmatic staining, with COX-2 light expression.

Progesterone Receptor Immunoreactivity

PR expression was detected by semiquantitative scoring in 14 of 31 cases in study (45.16%), all 14 cases had at least 50% of PR expression and all cases had a moderate or strong staining intensity. Immunostaining intensity was negative in 17 cases, moderate in 10 cases and strong in 4 cases. In figure 11 it can be seen a strong immunostaining for PR with strong expression.

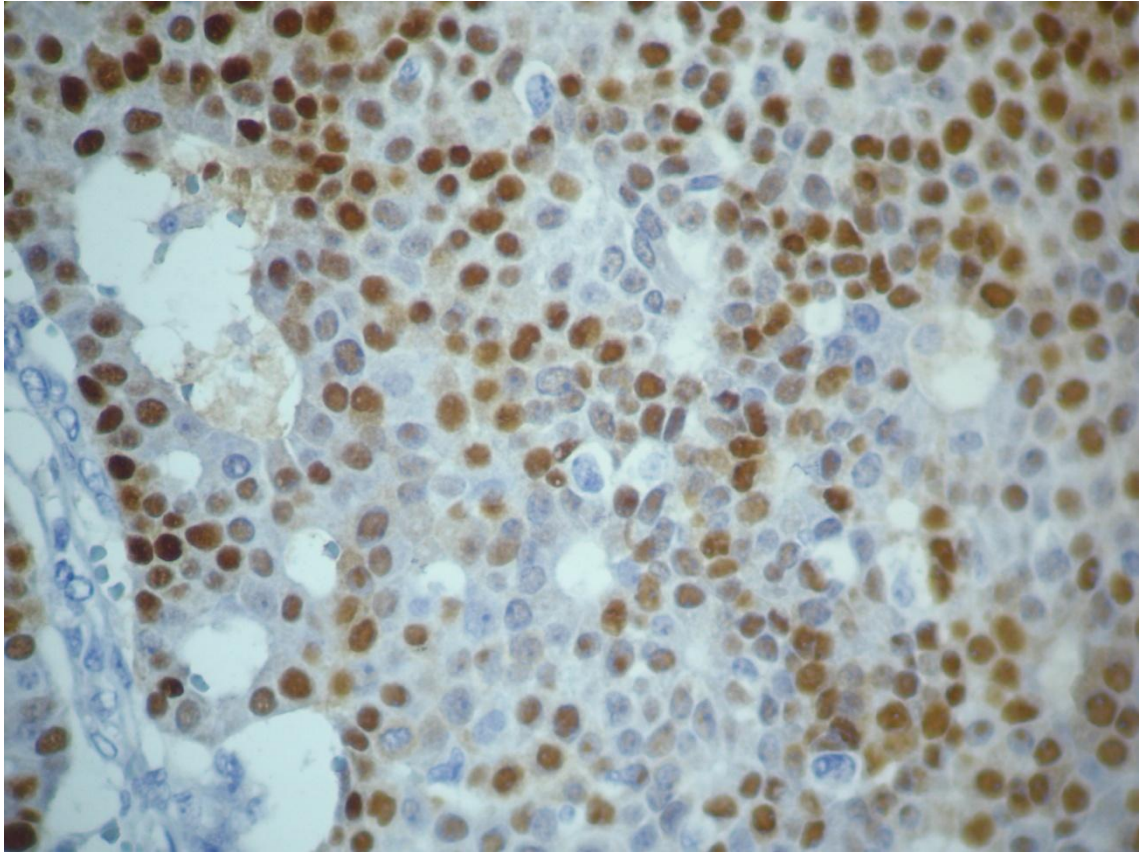


Figure 11: Immunohistochemical localization of PR in ductal carcinoma (400x magnification). There is nuclear strong staining, with PR high expression.

Comparative Statistical analysis

Correlation between Clinicopathologic Factors and Cyclooxygenase-2 Expression

As described previously, 20 (64.52%) cases in study were positive for COX-2 expression and 35.48% were negative.

When COX-2 expression was correlated with age, it was verified that positive COX-2 expression group is more heterogeneous, have a higher standard deviation (16.027 years) than negative cases (8.251 years) (see annex 2).

Minimum age and maximum age are respectively 39 and 87 years old for positive COX-2 expression group, and 59 and 91 years, for negative COX-2 expression group. Breast cancers positive for COX-2 expression also have a non-statistical significant and lower mean (65.65 years) than breast cancers negative for COX-2 expression (75.45 years). Positive cases for COX-2 expression seem to appear in earlier ages than negative cases (see figure 12).

However, there is no statistic significance ($p = 0.121$) when COX-2 expression is correlated with age.

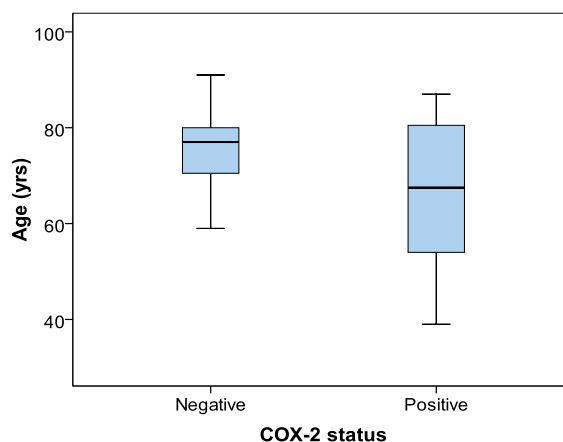


Figure 12: Correlation between COX-2 expression and age. For COX-2 negative expression the minimum value is 59 years and the maximum value is 91 years, with a mean of 75.45 years and a standard deviation of 8.251 years. For COX-2 positive expression the minimum value is 39 years and the maximum value is 87 years, with a mean of 65.65 years and a standard deviation of 16.027 years.

No statistic significance was found between tumor size and COX-2 positivity ($p = 0.725$). Minimum value for tumor size was the same in both positive and negative COX-2 groups (1 cm). The tumor size mean values for both groups are very similar, 3.95 cm for negative and 3.36 cm for positive COX-2 group (see annex 3). Standard deviation values were also closest, 2.709 cm and 2.952 cm for negative and positive COX-2 group (see figure 13).

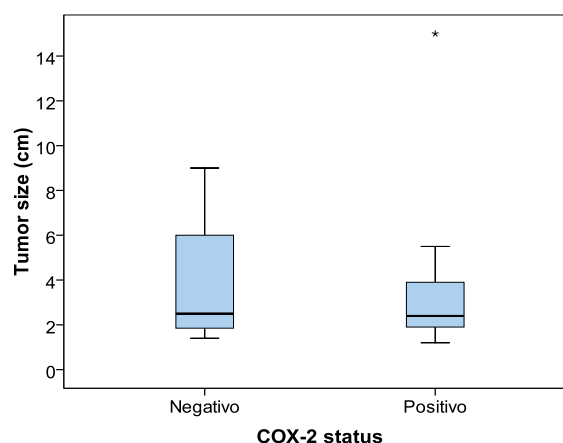


Figure 13: Correlation between COX-2 expression and tumor size. For COX-2 negative expression the minimum value is 1 cm and the maximum value is 9 cm, with a mean of 3.95 cm and a standard deviation of 2.709 cm. For COX-2 positive expression the minimum value is 1cm and the maximum value is 15 cm, with a mean of 3.36 cm and a standard deviation of 2.952 cm.

Even considering 2 groups of breast cancer (see figure 14), one with better prognosis tumors, with less than 2 cm (T1) and other with worst prognostic disease, with size of 2 or more cm, it was not found statistical significance between tumor size and COX-2 immunopositivity ($p = 0.510$) (see annex 4).

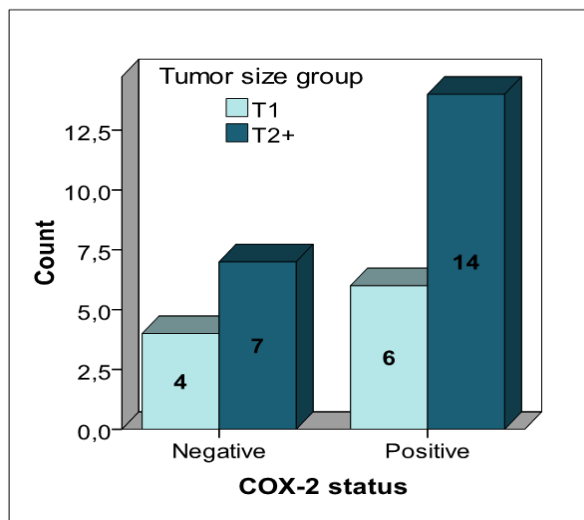


Figure 14: Correlation between COX-2 expression and age, considering 2 groups. For COX-2 negative expression there are 4 tumors with less than 2 cm and 7 tumors with 2cm or more. For COX-2 positive expression there are 6 cases with less than 2 cm and 14 cases with 2 cm or more.

Tumor histologic grade was considered as low risk for G1 tumors and high risk for G2 and G3 tumors. Of G1 histologic grade was recorded in 22.6% cases and 77.4% was classified as G2 and G3 histologic grade (see annex 5). Six cases (30.0%) with G1 histologic grade and 14 cases (70.0%) with G2 and G3 histologic grade were reported as positive for COX-2 immunostaining. Negative COX-2 expression group was found in 1 case (9.1%) with G1 histologic grade and in 10 cases (90.9%) with high G2 or G3 histologic grade (see figure 15).

There was not found any statistical significance correlation between COX-2 immunopositivity and tumor histological grade ($p = 0.192$).

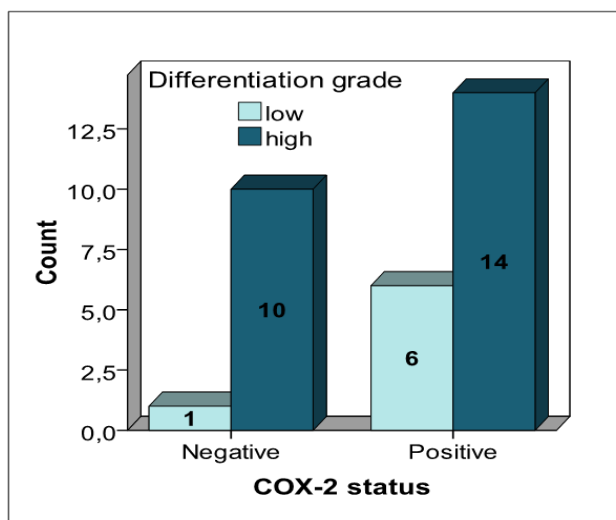


Figure 15: Correlation between COX-2 expression and differentiation grade. For COX-2 negative expression there is 1 tumor with low differentiation grade and 10 tumors with G2 or G3 histologic grade. For COX-2 positive expression there are 6 tumors with low differentiation grade and 14 tumors with G2 or G3 histologic grade.

Axillary node metastasis were documented in 9 of 31 cases (29.0%) the percentage of cases positive and negative for axillary node metastasis (54.5%) were very similar (45.5%) in those negative COX-2 tumors (see annex 6). In positive COX-2 cases the results are much different; there were 16 cases with no axillary node metastasis (80%) and 4 cases with axillary node metastasis (20%) (see figure 16). However, statistical analysis have not showed significance between COX-2 positivity and axillary node metastasis ($p = 0.140$).

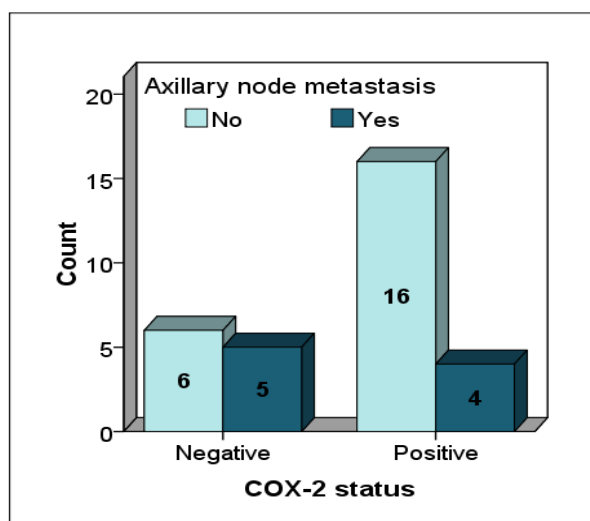


Figure 16: Correlation between COX-2 expression and axillary node metastasis. In 16 of the 20 women whose tumors overexpressed COX-2 there was no axillary node metastasis and in 4 of them there was axillary node metastasis. In tumors with negative COX-2 expression, 6 of 11 women had no axillary node metastasis and 5 of them had axillary node metastasis.

Correlation between Clinicopathologic Factors and Progesterone Receptor Expression

Fourteen cases were considered immunopositive for PR and 17 tumors were negative.

The age of the patients was not statistically correlated with positivity for PR (see annex 7).

The minimum age of the patients with tumors negative and positive to PR was the same, however, the group of the patients with tumors positive for PR expression, seemed more heterogeneous in considering the age, with a higher mean and higher standard deviation age (see figure 17).

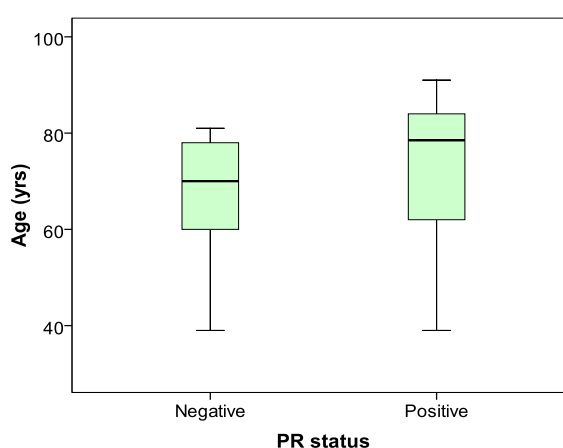


Figure 17: Correlation between PR expression and age. For PR negative expression the minimum value is 39 years and the maximum value is 81 years, with a mean of 67.59 years and a standard deviation of 11.609 years. For PR positive expression the minimum value is 39 years and the maximum value is 91 years, with a mean of 71 years and a standard deviation of 17.537 years.

Negative PR group had a higher mean (4.60 cm) and higher standard deviation (3.471 cm) than positive PR expression group which has a mean value of 2.32 cm and a standard deviation value of 0.816 cm (see figure 18). Negative PR expression group have tumors with higher size than positive PR expression group and is a more heterogeneous group than positive PR expression group.

The PR positive tumors were statistically significant smaller than PR negative tumors (see annex 8). There is statistical significance ($p = 0.032$).

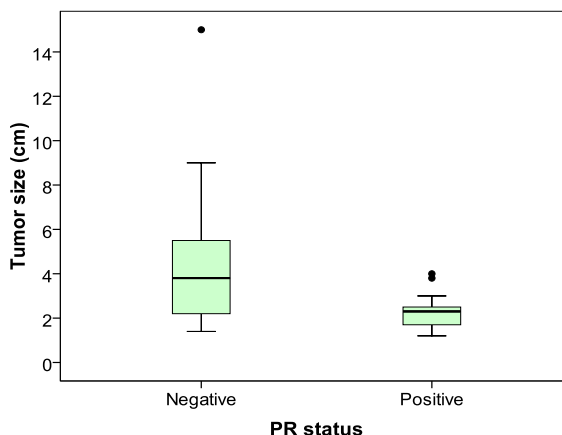


Figure 18: Correlation between PR expression and tumor size. For PR negative expression the minimum value is 1 cm and the maximum value is 15 cm, with a mean of 4.60 cm and a standard deviation of 3.471 cm. For PR positive expression the minimum value is 1 cm and the maximum value is 4 cm, with a mean of 2.32 cm and a standard deviation of 0.816 cm.

As reported earlier tumor differentiation grade was considered as low risk (G1) or high risk (G2 or G3) and it was correlated with PR expression groups.

In negative PR group 15 of 17 cases (88.2%) had G2 or G3 differentiation grade and in positive PR group 9 of 14 cases (64.3%) had G2 and G3 histologic grade (see figure 19).

When PR immunostaining is negative it seems that tumors tend to be a higher risk histologic grade, however, there is no statistical significance ($p = 0.124$) (see annex 9).

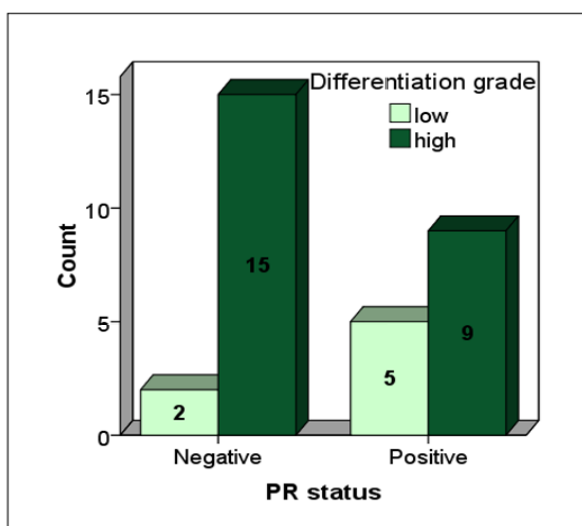


Figure 19: Correlation between PR expression and differentiation grade. For PR negative expression there are 2 tumors with low differentiation grade and 15 tumors with G2 or G3 histologic grade. For PR positive expression there are 5 tumors with low differentiation grade and 9 tumors with G2 or G3 histologic grade.

In tumors with immunopositivity for PR there were 13 of 14 (92.9%) cases without axillary nodes metastasis (see figure 20). PR negative immunostaining was strongly statistically associated with presence of axillary node metastasis ($p = 0.018$) (see annex 10).

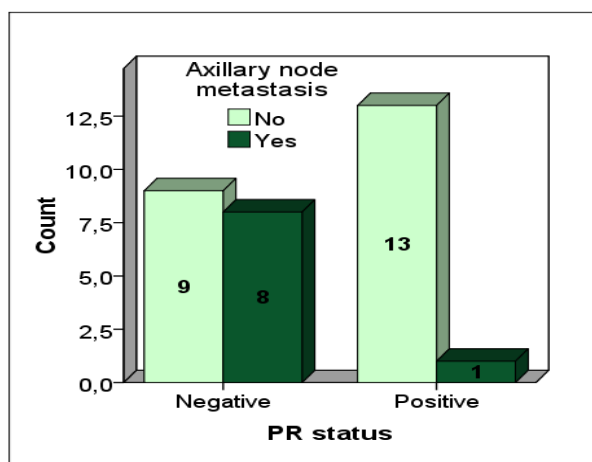


Figure 20: Correlation between PR expression and axillary node metastasis. In 13 of women whose tumors expressed PR there was no axillary node metastasis and in 1 of them there was axillary node metastasis. In tumors with negative PR expression, 9 women had no axillary node metastasis and 8 of them had axillary node metastasis.

Correlation between Progesterone Receptor Expression and Clinicopathologic Factors according to Cyclooxygenase-2 Expression

In order to evaluate any association between PR and COX-2 immunoexpression it was constituted 4 groups: positive COX-2 expression with positive PR expression ($COX-2^+/PR^+$), positive COX-2 expression with negative PR expression ($COX-2^+/PR^-$), negative COX-2 expression with positive PR expression ($COX-2^-/PR^+$) and negative COX-2 expression with negative PR expression ($COX-2^-/PR^-$).

$COX-2^+/PR^+$ group is constituted for 11 of 31 cases (35.48%), $COX-2^+/PR^-$ group is constituted for 9 of 31 cases (29.03%), $COX-2^-/PR^+$ group is constituted for 3 of 31 cases (9.68%) and $COX-2^-/PR^-$ group is constituted for 8 of 31 cases (25.81%) (see table 2).

Table 2: Correlation between COX-2 and PR expression.

	COX-2 negative expression	COX-2 positive expression	Total
PR negative expression	n = 8 (25.81%)	n = 9 (29.03%)	n = 17 (54.84%)
PR positive expression	n = 3 (9.68%)	n = 11 (35.48%)	n = 14 (45.16%)
Total	n = 11 (35.49%)	n = 20 (64.51%)	31 (100%)

As it can be seen in figure 21 a) and b), in patient age analysis, the group COX-2⁻/PR⁺ is the more heterogeneous and showed the higher value. Patients in the group COX-2⁻/PR⁻ seems to be the more aged with mean age of 83 years (see annex 11).

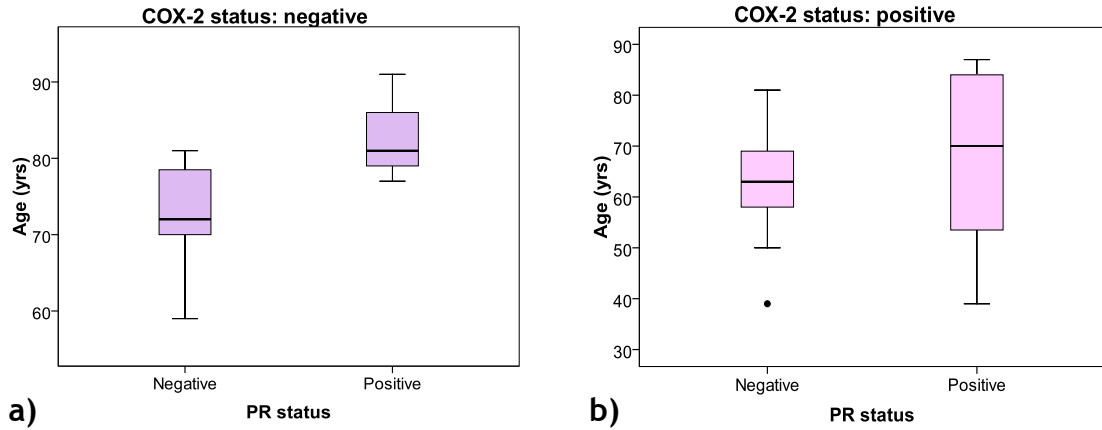


Figure 21: Correlation between PR expression and age according to COX-2 expression. a) The COX-2⁻/PR⁻ group is more heterogeneous than COX-2⁻/PR⁺ group, with a standard deviation of 6.989 years. COX-2⁻/PR⁺ has a higher mean and median than COX-2⁻/PR⁻ group. b) COX-2⁺/PR⁺ group has a higher mean and standard deviation than COX-2⁺/PR⁻ group

Evaluating tumor size, the COX-2⁻/PR⁻ group has shown to be the more heterogeneous (see figure 22a), ranging the size from 10 mm to 90 mm, and the group COX-2⁻/PR⁺ the more homogeneous (see annex 12). Comparing COX-2 positive expression group it was found that the size of the COX-2⁺/PR⁺ were statistically smaller that COX-2⁺/PR⁻ group (see figure 22b), there is statistical significance ($p = 0.043$).

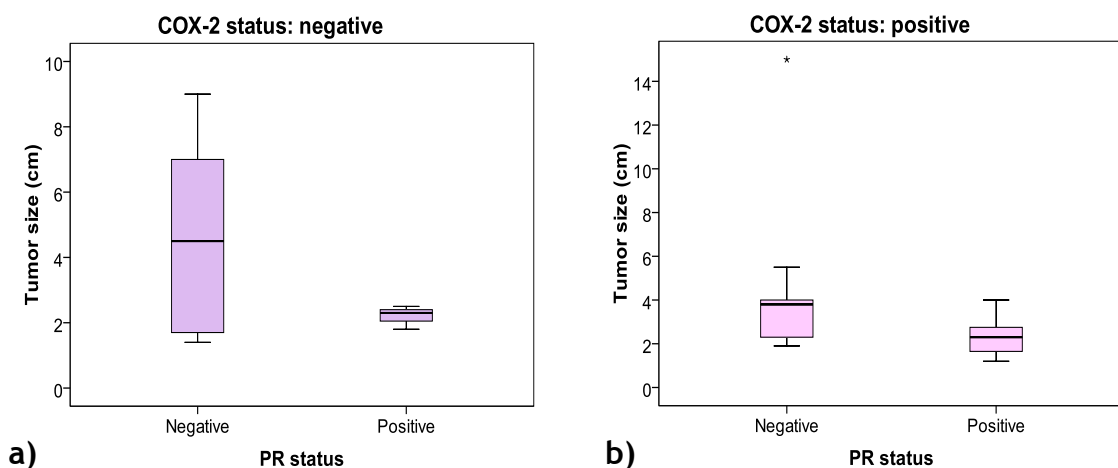


Figure 22: Correlation between PR expression and tumor size according to COX-2 expression. a) The COX-2⁻/PR⁻ group is more heterogeneous and has higher mean than COX-2⁻/PR⁺ group, with a standard deviation of 2.941 cm and a mean of 4.60 cm. b) COX-2⁺/PR⁺ group has a lower mean and standard deviation than COX-2⁺/PR⁻ group.

There are 10 cases with high differentiation grade, 8 of them belong to COX-2⁻/PR⁻ group and in this group there is no low differentiation grade, the number of high differentiation grade represents 100% (see figure 23 a and b). For COX-2⁻/PR⁺ group there are 2 cases with high differentiation grade and 1 case with low differentiation grade, which represents 66.7% and 33.3%, respectively (see annex 13).

For COX-2⁺/RP⁻ group there are 2 of 9 cases (22.2%) with low differentiation grade and 7 of 9 cases (77.8%) with high differentiation grade. Similar results were obtained for COX-2⁺/PR⁺ group, 4 of 11 cases (26.4%) have low differentiation grade and 7 of 11 cases (63.6%) have high differentiation grade.

All groups have more cases of high differentiation grade, and there is no statistical significance for any group. Negative COX-2 expression group has a P-value = 0.273 and positive COX-2 expression group has a P-value = 0.426.

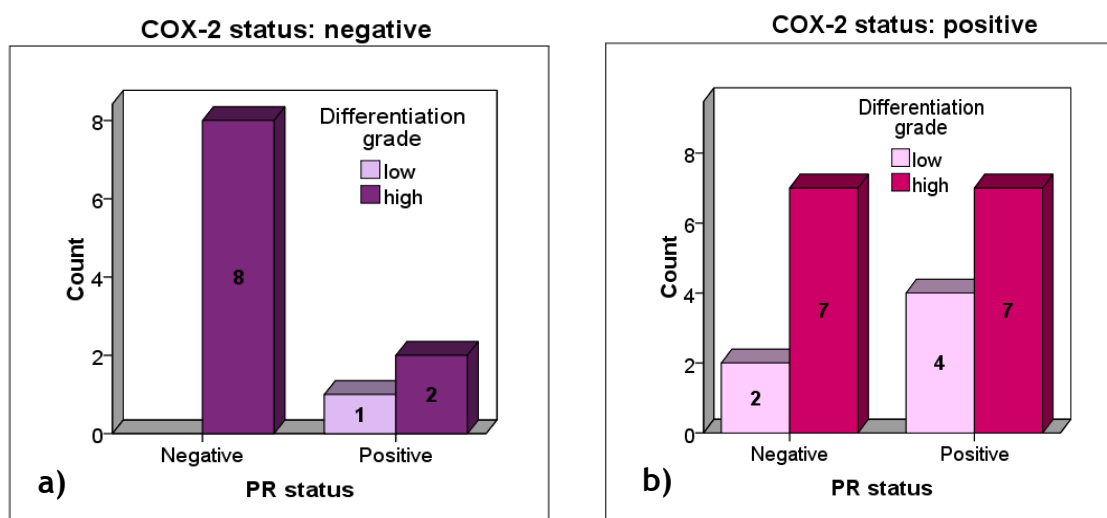


Figure 23: Correlation between PR expression and differentiation grade according to COX-2 expression. a) In COX-2⁻/PR⁻ group all tumors have a high differentiation grade and in COX-2⁻/PR⁺ group there is only one case with low differentiation grade. b) For COX-2 positive expression group tumors with PR positive expression have higher number of cases with low differentiation grade (G1).

For negative COX-2 expression group 6 of 11 (54.55%) women had no axillary node metastasis (see annex 14).

In COX-2⁻/PR⁻ group 50% of women had axillary node metastasis and in COX-2⁻/PR⁺ group there was only 1 woman with axillary node metastasis (33.3%) (see figure 24a). For negative COX-2 expression group there was not found statistical significance (P-value = 0.576).

For positive COX-2 expression group there are 16 of 20 women (80%) without axillary node metastasis and 4 of 20 women (20%) with axillary node metastasis (see figure 24b).

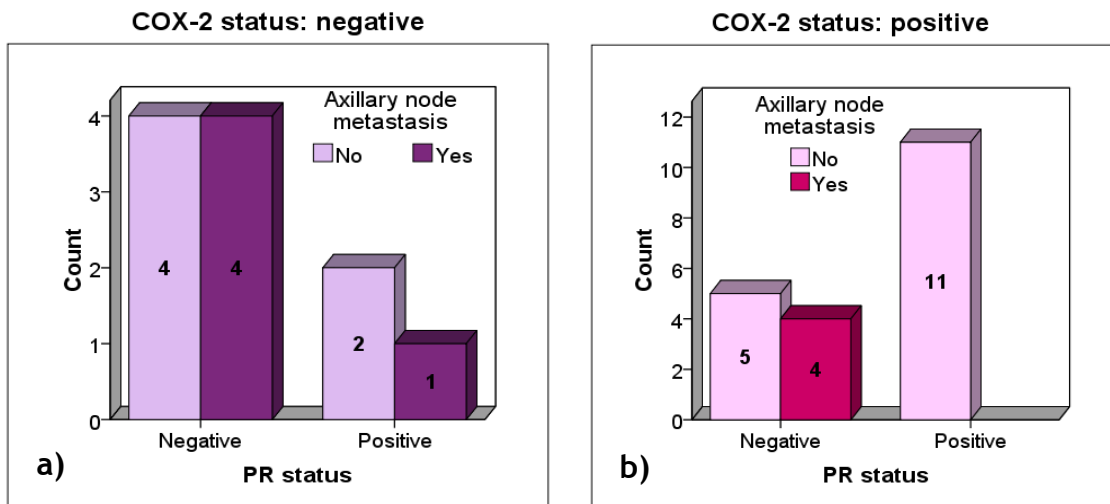


Figure 24: Correlation between PR expression and axillary node metastasis according to COX-2 expression. a) In COX-2⁻/PR⁻ there is the same number of cases with and without axillary node metastasis, for PR negative expression there are more cases without axillary node metastasis. b) COX-2⁺/PR⁻ has a higher number of cases with axillary node metastasis than COX-2⁺/PR⁺ group, which has only cases without axillary node metastasis.

There were nine women whose tumors were COX-2⁺/PR⁻, and 5 (55.6%) of them had no axillary node metastasis. In COX-2⁺/PR⁺ group all women (100%) had no axillary node metastasis. These groups are statistical related (P-value = 0.026).

Chapter 4: Discussion

COX-2 is the induced cyclooxygenase isoform, is expressed in most of the tissues and commonly expressed in more than 50% of invasive breast cancers (Haffty et al. 2008; Singh-Ranger et al. 2008). COX-2 is involved in chronic inflammation and is responsible for AA conversion into prostaglandins, which are associated with increased aromatase that in turn, results in high local ER (Mendelson and Hardy 2006; Schetter et al. 2010).

ER upregulation is associated with breast cancer progression and PR has been considered has an estrogen-induced target gene (Cui et al. 2005). However, Hardy et al. found that PR expression is an independent predictor of breast cancer diagnosis (Hardy et al. 2008). PR is a COX-2 and aromatase inhibitor and plays an important anti-inflammatory and protective role in breast cancer (Hardy et al. 2008).

Based on these findings we attempted to determine a relation between COX-2 expression, PR expression and clinicopathologic factors (age, tumor size, histologic grade and axillary node metastasis), which are known to be prognostic factors.

As previously reported, our study was performed in 31 women with invasive ductal breast cancer and COX-2 expression was detected by semiquantitative scoring in 64.52% of tumors. These results are consistent with those of Haffty et al., who found COX-2 expression in 58% of women (n = 504) with breast cancer (Haffty et al. 2008).

When we performed a comparative study between COX-2 expression and age, we observed that the tumors with COX-2 expression had a tendency for incidence in younger ages, once all women with 50 years or less (n=5) had COX-2 expression. However, that results have shown no statistical significance ($p = 0.121$) probably due to the reduced number of cases. Haffty et al. found similar results: nearly 70% of women with less than 40 years of age expressed COX-2 and these results are consistent with those of Ristimäki et al. and Denkert et al. studies (Ristimäki et al. 2002; Denkert et al. 2003; Haffty et al. 2008).

Previous studies have also reported that tumors with COX-2 expression were higher than 2 cm (Ristimäki et al. 2002; Denkert et al. 2003; Haffty et al. 2008), however, in our study, COX-2 expression and tumor size were not statistical related ($p > 0.005$), probably due to the low number of cases in the study.

Correlating COX-2 expression with histologic grade and with axillary node metastasis we found no statistical association between them. The group of tumors that expressed COX-2 and the group of tumors that has no COX-2 expression had similar number of cases with high histologic grade. However, other researchers concluded that COX-2 expression is associated with high histologic grade (Ristimäki et al. 2002; Denkert et al. 2003). In our study axillary node

metastasis was negatively associated with COX-2 expression and this is inconsistent with previous studies. We found less than expected axillary node metastasis in the group of tumors with COX-2 expression, compared with the group of tumors that has no COX-2 expression. Ristimäki et al. and Denkert et al. obtained opposite results, there was a higher incidence of cases with axillary node metastasis when there was COX-2 expression, concluding that COX-2 expression is related to axillary node metastasis (Ristimäki et al. 2002; Denkert et al. 2003). Probably, one way to explain this results disparity may be the inclusion of different types of breast cancer in studies of Ristimäki et al. and Denkert et al. (Ristimäki et al. 2002; Denkert et al. 2003).

Clinicopathologic factors there were also statistical compared with PR expression. By semiquantitative scoring there was found that PR expression was present in 45.16% of the cases, being this result in according with previous studies (Lee et al. 2010).

In our study we found no statistical significance between PR expression and age and histologic grade, although, the group with negative PR expression has shown a tendency to higher incidence of tumors with high histologic grade.

We found statistical relation ($p = 0.032$) between tumor size and PR status. Tumors with smaller size had PR expression. Similar statistical significance were obtained when PR expression was compared with axillary node metastasis ($p = 0.018$). These results suggest that PR expression might have a protective role in breast cancer.

It was not possible to establish association between COX-2 expression and clinicopathologic factors, COX-2 expression seems to appear in younger patients, it is also possible to be related with COX-2 increased risk for axillary node metastasis. Several studies have been conducted in order to establish a relationship between COX-2 expression and clinicopathologic factors (Haffty et al. 2008). Ristimäki et al., Denkert et al. and Haffty et al. concluded that the COX-2 expression is related to tumor appearance at early ages, higher tumor size, histological grade and axillary lymph node metastasis (Ristimäki et al. 2002; Denkert et al. 2003; Haffty et al. 2008). But as in the present study, Lee et al. also found no significant correlation between COX-2 expression and clinicopathologic factors, probably due to the reduced data set (Lee et al. 2010).

In our study, we could establish a relation between PR and COX-2 expression with the studied clinicopathologic factors, but these results have no concordance with the ones found for Lee et al. who did not establish this correlation (Lee et al. 2010). Probably that discrepancy was due to the methodology used. They considered $>10\%$ PR or COX-2 of staining as a positive case and in our study only the cases with at least 50% of staining were considered as positive. Our methodology seems more reasonable in order to avoid false positive cases.

When both PR and COX-2 expression were compared with age, patients with tumors COX-2⁻/PR⁺ seems to be younger, although COX-2 expression, PR expression and age are not statistical related ($p > 0.05$).

Tumors COX-2⁺/PR⁺ were smaller that those with COX-2⁺/PR⁻ ($p = 0.043$). Comparing axillary node metastasis with COX-2 expression and PR expression we found that none of the patients whose tumors expressed PR had axillary node metastasis. As previously reported, PR is related with axillary node metastasis, taken this together with COX-2 expression, it is possible that PR may have a protective role in breast cancer.

Is known that PR positive breast cancer is associated with a better prognosis and negative PR expression is worse signal (Bardou et al. 2003). It was suggested benefits for chemotherapy in PR negative breast cancer, including small or intermediated size and node negative cancers, in according with bad prognosis of this kind of breast cancers (National Institutes of Health 2000).

Despite PR protective role in breast cancer is unclear, Hardy et al. verified in cellular breast cancer lines, that PR antagonizes COX-2 expression (Hardy et al. 2008). PR acts as a ligand dependent and independent way to inhibit NF- κ B (COX-2 transcriptional factor) transactivation (Mendelson and Hardy 2006; Hardy et al. 2008). PR interferes with COX-2 transactivation through NF- κ B, via I κ B α induction, which modulates COX-2 expression (Hardy et al. 2008).

COX-2 expression is related with increased expression of aromatase, and consequent local increase of ER, PR expression might decrease aromatase induction, with consequent decrease of local ER (Mendelson and Hardy 2006; Hardy et al. 2008).

Thus, decreased local ER levels occur as a result of decreased expression of COX-2 (Mendelson and Hardy 2006; Hardy et al. 2008).

This modulating role, played by PR can be one of the reasons to the ER⁺/PR⁻ hormone tumors are more aggressive and have higher proliferation rates when compared with ER⁻/PR⁺ tumors (Arpino et al. 2005). ER⁺/PR⁻ tumors are more resistant to adjuvant treatment, specially to tamoxifen and endocrine therapy, progesterone treatment may be an important consideration (Bardou et al. 2003; Arpino et al. 2005).

Thus, high levels of PR in breast epithelial cells play a protective role in breast cancer, by modulating inflammatory response pathway. Despite have been provided evidences that there is a relationship between PR lower expression, COX-2 expression and aggressiveness of breast cancer, further studies are needed to clarify all the mechanisms by which PR plays an inflammatory role in this type of cancer.

Our results suggest that ductal invasive breast cancer PR positive may have different prognosis in according of COX-2 expression. In fact, those cancers COX-2⁺/PR⁺ seems to have different behavior of COX-2⁺/PR⁻, and COX-2⁺/PR⁺ immunopexpression represents a marker for better prognosis. We speculate if COX-2 expression will be related with better disease-free survival and overall-survival in patients immunopositive for PR submitted to adjuvant hormonal therapy.

Further studies may clarify the usefulness of the COX-2 immunostaining in clinical practice.

Chapter 5: Conclusion

The purpose for this study was to perform a correlation between COX-2 expression, PR expression and clinicopathologic factors in breast cancer, through an immunostaining evaluation.

There was found a positive association between PR expression between breast tumor size and axillary node metastasis.

COX-2⁺/PR⁺ was associated with lower tumor size and lower incidence of axillary node metastasis.

These findings suggest that PR modulates COX-2 expression and its expression is related with better prognosis. Conversely, a decline PR expression is associated with an overall worse survival.

COX-2 seems to be a marker for good behaviour in ductal invasive breast cancer.

We speculate if COX-2 determination may have be a clinical usefulness in clinical practice. It's expected that further studies may clarify this issue.

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Annexes

Annex 1

Patients Data

Patients	Age	Year	COX-2 immuno.	COX-2 exp.	PR immuno.	PR exp.	Tumor size	Diff. Grad.	Axillary node metast.
1	39	2007	+	50%	+	50%	16mm -2	G2	Negative
2	43	2009	+	50%	+	75%	23mm -2	G3	Negative
3	45	2007	+	75%	+	50%	12mm -1	G2	Negative
4	66	2007	+	50%	+	50%	19mm -2	G3	Negative
5	67	2007	++	100%	++	100%	16mm -2	G1	Negative
6	70	2009	+	50%	+	75%	23mm -2	G2	Negative
7	80	2009	+	50%	+	50%	25mm -3	G2	Negative
8	84	2009	+	50%	+	75%	40mm	G2	Negative
9	84	2009	+	50%	+	50%	38mm -4	G1	Negative
10	85	2009	+	75%	+	100%	17mm -2	G1	Negative
11	87	2009	+	75%	+	75%	30mm	G1	Negative
12	39	2007	+	50%	-	25%	40mm	G3	Negative
13	50	2009	+	50%	-	25%	40mm	G2	Positive
14	58	2008	+	75%	-	25%	38mm -4	G2	Negative
15	60	2008	+	50%	-	0	19mm -2	G3	Negative
16	63	2009	+	50%	-	0	55mm -6	G3	Positive
17	69	2007	+	50%	-	25%	23mm -2	G2	Positive
18	69	2009	+	50%	-	0	27mm -3	G1	Negative
19	79	2007	+	50%	-	0	150mm	G1	Positive
20	81	2007	++	75%	-	0	22mm	G2	Negative
21	77	2009	-	0	++	75%	25mm -3	G1	Negative
22	81	2008	-	25%	++	100%	18mm -2	G2	Negative
23	91	2007	-	25%	++	75%	23mm -2	G2	Positive
24	59	2007	-	25%	-	0	15mm -2	G3	Negative
25	70	2007	-	25%	-	0	19mm -2	G2	Negative

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26	70	2009	-	25%	-	0	90mm	G3	Positive
27	71	2009	-	25%	-	0	75mm ~8	G2	Positive
28	73	2007	-	25%	-	0	14mm ~1	G2	Positive
29	78	2009	-	25%	-	0	35mm ~4	G2	Negative
30	79	2008	-	25%	-	0	55mm ~6	G2	Positive
31	81	2007	-	0	-	0	65mm ~7	G3	Negative

Patients; Age; Year; COX-2 immunostaining; COX-2 expression; PR immunostaining; PR expression; Tumor size; Differentiation Grade; Axillary node metastasis

Annex 2

Correlation between Cyclooxygenase-2 Expression and Age

Ranks				
	COX-2 status	N	Mean Rank	Sum of Ranks
Age (yrs)	Negative	11	19,41	213,50
	Positive	20	14,13	282,50
	Total	31		

Test Statistics ^b	
	Age
Mann-Whitney U	72,500
Wilcoxon W	282,500
Z	-1,550
Asymp. Sig. (2-tailed)	,121
Exact Sig. [2*(1-tailed Sig.)]	,123 ^a
a. Not corrected for ties.	
b. Grouping Variable: COX-2 status	

Case Processing Summary							
	COX-2 status	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Age (yrs)	Negative	11	100,0%	0	,0%	11	100,0%
	Positive	20	100,0%	0	,0%	20	100,0%

Descriptives					
	COX-2 status		Statistic	Std. Error	
Age (yrs)	Negative	Mean		75,45	2,488
		95% Confidence Interval for Mean	Lower Bound	69,91	
			Upper Bound	81,00	
		5% Trimmed Mean		75,51	
		Median		77,00	
		Variance		68,073	
		Std. Deviation		8,251	
		Minimum		59	
		Maximum		91	
		Range		32	
		Interquartile Range		11	
		Skewness		-,157	,661
		Kurtosis		1,214	1,279
	Positive	Mean		65,65	3,584
		95% Confidence Interval for Mean	Lower Bound	58,15	
			Upper Bound	73,15	
		5% Trimmed Mean		65,94	
		Median		67,50	
		Variance		256,871	
		Std. Deviation		16,027	
		Minimum		39	
		Maximum		87	
		Range		48	
		Interquartile Range		29	
		Skewness		-,346	,512
Kurtosis		-1,105	,992		

Annex 3

Correlation between Cyclooxygenase-2 Expression and Tumor Size

Ranks				
	COX-2 status	N	Mean Rank	Sum of Ranks
Tumor Size (cm)	Negative	11	16,77	184,50
	Positive	20	15,58	311,50
	Total	31		

Test Statistics ^b	
	Tumor size
Mann-Whitney U	101,500
Wilcoxon W	311,500
Z	-,352
Asymp. Sig. (2-tailed)	,725
Exact Sig. [2*(1-tailed Sig.)]	,730 ^a
a. Not corrected for ties.	
b. Grouping Variable: COX-2 status	

Case Processing Summary							
	COX-2 status	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Tumor size (cm)	Negative	11	100,0%	0	,0%	11	100,0%
	Positive	20	100,0%	0	,0%	20	100,0%

Descriptives					
	COX-2 status		Statistic	Std. Error	
Tumor size (cm)	Negative	Mean	3,95	,817	
		95% Confidence Interval for Mean	Lower Bound	2,13	
			Upper Bound	5,77	
		5% Trimmed Mean	3,81		
		Median	2,50		
		Variance	7,337		
		Std. Deviation	2,709		
		Minimum	1		
		Maximum	9		
		Range	8		
		Interquartile Range	5		
		Skewness	,845	,661	
		Kurtosis	-,798	1,279	
	Positive	Mean	3,36	,660	
		95% Confidence Interval for Mean	Lower Bound	1,98	
			Upper Bound	4,75	
		5% Trimmed Mean	2,84		
		Median	2,40		
		Variance	8,715		
		Std. Deviation	2,952		
		Minimum	1		
		Maximum	15		
		Range	14		
		Interquartile Range	2		
Skewness	3,517	,512			
Kurtosis	13,973	,992			

Annex 4

Correlation between Cyclooxygenase-2 Expression and Tumor Size Group

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
COX-2 status * Tumor size group	31	100,0%	0	,0%	31	100,0%

Crosstab					
		Tumor size group			
			T1	T2+	Total
COX-2 status	Negative	Count	4	7	11
		% within COX-2 status	36,4%	63,6%	100,0%
		% within Tumor size group	40,0%	33,3%	35,5%
		% of Total	12,9%	22,6%	35,5%
	Positive	Count	6	14	20
		% within COX-2 status	30,0%	70,0%	100,0%
		% within Tumor size group	60,0%	66,7%	64,5%
		% of Total	19,4%	45,2%	64,5%
Total		Count	10	21	31
		% within COX-2 status	32,3%	67,7%	100,0%
		% within Tumor size group	100,0%	100,0%	100,0%
		% of Total	32,3%	67,7%	100,0%

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	,132 ^a	1	,717		
Continuity Correction ^b	,000	1	1,000		
Likelihood Ratio	,130	1	,718		
Fisher's Exact Test				1,000	,510
Linear-by-Linear Association	,127	1	,721		
N of Valid Cases	31				
a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 3,55.					
b. Computed only for a 2x2 table					

Risk Estimate			
	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for COX-2 status (Negative / Positive)	1,333	,281	6,325
For cohort Tumor size group = T1	1,212	,433	3,393
For cohort Tumor size group = T2+	,909	,535	1,546
N of Valid Cases	31		

Annex 5

Correlation between Cyclooxygenase-2 expression and Differentiation Grade

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
COX-2 status * Differentiation grade	31	100,0%	0	,0%	31	100,0%

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
COX-2 status * Differentiation grade	31	100,0%	0	,0%	31	100,0%
COX-2 status * Axillary node metastasis	31	100,0%	0	,0%	31	100,0%

Crosstab					
			Differentiation grade		Total
			1	2 or 3	
COX-2 status	Negative	Count	1	10	11
		% within COX-2 status	9,1%	90,9%	100,0%
		% within Differentiation grade	14,3%	41,7%	35,5%
		% of Total	3,2%	32,3%	35,5%
	Positive	Count	6	14	20
		% within COX-2 status	30,0%	70,0%	100,0%

		% within Differentiation grade	85,7%	58,3%	64,5%
		% of Total	19,4%	45,2%	64,5%
Total		Count	7	24	31
		% within COX-2 status	22,6%	77,4%	100,0%
		% within Differentiation grade	100,0%	100,0%	100,0%
		% of Total	22,6%	77,4%	100,0%

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1,775 ^a	1	,183		
Continuity Correction ^b	,780	1	,377		
Likelihood Ratio	1,981	1	,159		
Fisher's Exact Test				,372	,192
Linear-by-Linear Association	1,718	1	,190		
N of Valid Cases	31				
a. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 2,48.					
b. Computed only for a 2x2 table					

Risk Estimate			
	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for COX-2 status (Negative / Positive)	,233	,024	2,252
For cohort Differentiation grade= 1	,303	,042	2,206
For cohort Differentiation grade = 2 ou 3	1,299	,922	1,829
N of Valid Cases	31		

Annex 6

Correlation between Cyclooxygenase-2 Expression and Axillary Node Metastasis

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
COX-2 status * Axillary node metastasis	31	100,0%	0	,0%	31	100,0%

Crosstab					
			Ganglion metastasis		Total
			No	Yes	
COX-2 status	Negative	Count	6	5	11
		% within COX-2 status	54,5%	45,5%	100,0%
		% within Ganglion metastasis	27,3%	55,6%	35,5%
		% of Total	19,4%	16,1%	35,5%
	Positive	Count	16	4	20
		% within COX-2 status	80,0%	20,0%	100,0%
		% within Ganglion metastasis	72,7%	44,4%	64,5%
		% of Total	51,6%	12,9%	64,5%
Total		Count	22	9	31
		% within COX-2 status	71,0%	29,0%	100,0%
		% within Ganglion metastasis	100,0%	100,0%	100,0%
		% of Total	71,0%	29,0%	100,0%

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2,232 ^a	1	,135		
Continuity Correction ^b	1,167	1	,280		
Likelihood Ratio	2,177	1	,140		
Fisher's Exact Test				,217	,140
Linear-by-Linear Association	2,160	1	,142		
N of Valid Cases	31				
a. 1 cells (25,0%) have expected count less than 5. The minimum expected count is 3,19.					
b. Computed only for a 2x2 table					

Risk Estimate			
	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for COX-2 status (Negative / Positive)	,300	,060	1,509
For cohort Ganglion metastasis = No	,682	,381	1,221
For cohort Ganglion metastasis = yes	2,273	,764	6,757
N of Valid Cases	31		

Annex 7

Correlation between Progesterone Receptor Expression and Age

Ranks				
	PR status	N	Mean Rank	Sum of Ranks
Age (yrs)	Negative	17	14,15	240,50
	Positive	14	18,25	255,50
	Total	31		

Test Statistics ^b	
	Age
Mann-Whitney U	87,500
Wilcoxon W	240,500
Z	-1,252
Asymp. Sig. (2-tailed)	,211
Exact Sig. [2*(1-tailed Sig.)]	,215 ^a
a. Not corrected for ties.	
b. Grouping Variable: 4. Rp %	

Case Processing Summary							
	PR status	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Age (yrs)	Negative	17	100,0%	0	,0%	17	100,0%
	Positive	14	100,0%	0	,0%	14	100,0%

Case Processing Summary							
	PR status	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Tumor size (cm)	Negative	17	100,0%	0	,0%	17	100,0%
	Positive	14	100,0%	0	,0%	14	100,0%

Descriptives						
	PR status			Statistic	Std. Error	
Age (yrs)	Negative	Mean		67,59	2,815	
		95% Confidence Interval for Mean	Lower Bound		61,62	
			Upper Bound		73,56	
		5% Trimmed Mean		68,43		
		Median		70,00		
		Variance		134,757		
		Std. Deviation		11,609		
		Minimum		39		
		Maximum		81		
		Range		42		
		Interquartile Range		19		
		Skewness		-,982	,550	
		Kurtosis		,820	1,063	
		Positive	Mean		71,00	4,687
	95% Confidence Interval for Mean		Lower Bound		60,87	
			Upper Bound		81,13	
	5% Trimmed Mean		71,67			
	Median		78,50			
	Variance		307,538			
	Std. Deviation		17,537			
	Minimum		39			
	Maximum		91			
	Range		52			
	Interquartile Range		27			
	Skewness		-,865	,597		
	Kurtosis		-,650	1,154		

Annex 8

Correlation between Progesterone Receptor Expression and Tumor Size

Ranks				
	PR status	N	Mean Rank	Sum of Ranks
Tumor size (cm)	Negative	17	19,18	326,00
	Positive	14	12,14	170,00
	Total	31		

Test Statistics ^b	
	Tumor size
Mann-Whitney U	65,000
Wilcoxon W	170,000
Z	-2,148
Asymp. Sig. (2-tailed)	,032
Exact Sig. [2*(1-tailed Sig.)]	,032 ^a
a. Not corrected for ties.	
b. Grouping Variable: 4. Rp %	

Case Processing Summary							
	PR status	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Tumor size (cm)	Negative	17	100,0%	0	,0%	17	100,0%
	Positive	14	100,0%	0	,0%	14	100,0%

Descriptives					
	PR status		Statistic	Std. Error	
Tumor size (cm)	Negative	Mean		4,60	,842
		95% Confidence Interval for Mean	Lower Bound	2,82	
			Upper Bound	6,38	
		5% Trimmed Mean		4,20	
		Median		3,80	
		Variance		12,051	
		Std. Deviation		3,471	
		Minimum		1	
		Maximum		15	
		Range		14	
		Interquartile Range		4	
		Skewness		1,872	,550
		Kurtosis		4,205	1,063
		Positive	Mean		2,32
	95% Confidence Interval for Mean		Lower Bound	1,85	
			Upper Bound	2,79	
	5% Trimmed Mean		2,29		
	Median		2,30		
	Variance		,666		
	Std. Deviation		,816		
	Minimum		1		
	Maximum		4		
	Range		3		
	Interquartile Range		1		
Skewness		,935	,597		
Kurtosis		,378	1,154		

Annex 9

Correlation between Progesterone Receptor Expression and Differentiation Grade

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PR status *	31	100,0%	0	,0%	31	100,0%
Differentiation grade						

Crosstab					
			Differentiation grade		Total
			1	2 or 3	
PR status	Negative	Count	2	15	17
		% within PR status	11,8%	88,2%	100,0%
		% within Differentiation grade	28,6%	62,5%	54,8%
		% of Total	6,5%	48,4%	54,8%
	Positive	Count	5	9	14
		% within PR status	35,7%	64,3%	100,0%
		% within Differentiation grade	71,4%	37,5%	45,2%
		% of Total	16,1%	29,0%	45,2%
Total	Count	7	24	31	
	% within PR status	22,6%	77,4%	100,0%	
	% within Differentiation grade	100,0%	100,0%	100,0%	
	% of Total	22,6%	77,4%	100,0%	

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2,519 ^a	1	,112		
Continuity Correction ^b	1,335	1	,248		
Likelihood Ratio	2,554	1	,110		
Fisher's Exact Test				,198	,124
Linear-by-Linear Association	2,438	1	,118		
N of Valid Cases	31				
a. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 3,16.					
b. Computed only for a 2x2 table					

Risk Estimate			
	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for PR status (Negative / Positive)	,240	,038	1,506
For cohort Differentiation grade = 1	,329	,075	1,446
For cohort Differentiation grade = 2 ou 3	1,373	,895	2,104
N of Valid Cases	31		

Annex 10

Correlation between Progesterone Receptor Expression and Axillary Node Metastasis

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PR status * Axillary node metastasis	31	100,0%	0	,0%	31	100,0%

Crosstab						
				Axillary node metastasis		Total
				No	Yes	
PR status	Negative	Count		9	8	17
		% within PR status		52,9%	47,1%	100,0%
		% within Ganglion metastasis		40,9%	88,9%	54,8%
		% of Total		29,0%	25,8%	54,8%
	Positive	Count		13	1	14
		% within PR status		92,9%	7,1%	100,0%
		% within Ganglion metastasis		59,1%	11,1%	45,2%
		% of Total		41,9%	3,2%	45,2%
Total	Count		22	9	31	
	% within PR status		71,0%	29,0%	100,0%	
	% within Ganglion metastasis		100,0%	100,0%	100,0%	
	% of Total		71,0%	29,0%	100,0%	

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5,937 ^a	1	,015		
Continuity Correction ^b	4,158	1	,041		
Likelihood Ratio	6,638	1	,010		
Fisher's Exact Test				,021	,018
Linear-by-Linear Association	5,745	1	,017		
N of Valid Cases	31				
a. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 4,06.					
b. Computed only for a 2x2 table					

Risk Estimate			
	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for PR status (Negative / Positive)	,087	,009	,818
For cohort Ganglion metastasis = No	,570	,356	,913
For cohort Ganglion metastasis = Yes	6,588	,933	46,530
N of Valid Cases	31		

Annex 11

Correlation between Progesterone Receptor Expression and Age according to Cyclooxygenase-2 Expression

Ranks					
COX-2 status		PR status	N	Mean Rank	Sum of Ranks
Negative	Age (yrs)	Negative	8	4,94	39,50
		Positive	3	8,83	26,50
		Total	11		
Positive	Age (yrs)	Negative	9	9,06	81,50
		Positive	11	11,68	128,50
		Total	20		

Test Statistics ^b		
COX-2 status		Age (yrs)
Negative	Mann-Whitney U	3,500
	Wilcoxon W	39,500
	Z	-1,743
	Asymp. Sig. (2-tailed)	,081
	Exact Sig. [2*(1-tailed Sig.)]	,085 ^a
Positive	Mann-Whitney U	36,500
	Wilcoxon W	81,500
	Z	-,989
	Asymp. Sig. (2-tailed)	,323
	Exact Sig. [2*(1-tailed Sig.)]	,331 ^a
a. Not corrected for ties.		
b. Grouping Variable: 4. Rp %		

Case Processing Summary								
COX-2 status		RP status	Cases					
			Valid		Missing		Total	
			N	Percent	N	Percent	N	Percent
Negative	Age (yrs)	Negative	8	100,0%	0	,0%	8	100,0%
		Positive	3	100,0%	0	,0%	3	100,0%
Positive	Age (yrs)	Negative	9	100,0%	0	,0%	9	100,0%
		Positive	11	100,0%	0	,0%	11	100,0%

Descriptives						
COX-2 status		PR status		Statistic	Std. Error	
Negative	Age (yrs)	Negative	Mean		72,63	2,471
			95% Confidence Interval for Mean	Lower Bound	66,78	
				Upper Bound	78,47	
			5% Trimmed Mean		72,92	
			Median		72,00	
			Variance		48,839	
			Std. Deviation		6,989	
			Minimum		59	
			Maximum		81	
			Range		22	
			Interquartile Range		9	
			Skewness		-,875	,752
		Kurtosis		1,133	1,481	
		Positive	Mean		83,00	4,163
			95% Confidence Interval for Mean	Lower Bound	65,09	
				Upper Bound	100,91	
			5% Trimmed Mean		.	
			Median		81,00	
			Variance		52,000	
			Std. Deviation		7,211	
			Minimum		77	
			Maximum		91	
Range			14			
Interquartile Range		.				

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			Skewness		1,152	1,225			
			Kurtosis		.	.			
Positive	Age (yrs)	Negative	Mean		63,11	4,458			
			95% Confidence Interval for Mean	Lower Bound		52,83			
				Upper Bound		73,39			
			5% Trimmed Mean		63,46				
			Median		63,00				
			Variance		178,861				
			Std. Deviation		13,374				
			Minimum		39				
			Maximum		81				
			Range		42				
			Interquartile Range		20				
			Skewness		-,423	,717			
			Kurtosis		-,077	1,400			
			Positive			Mean		67,73	5,514
		95% Confidence Interval for Mean				Lower Bound		55,44	
						Upper Bound		80,01	
		5% Trimmed Mean					68,25		
		Median					70,00		
		Variance					334,418		
		Std. Deviation					18,287		
		Minimum					39		
		Maximum					87		
		Range					48		
		Interquartile Range		39					
Skewness		-,559	,661						
Kurtosis		-1,359	1,279						

Annex 12

Correlation between Progesterone Receptor Expression and Tumor Size according to Cyclooxygenase-2 Expression

Ranks					
COX-2 status		PR status	N	Mean Rank	Sum of Ranks
	Tumor size (cm)	Negative	8	6,50	52,00
		Positive	3	4,67	14,00
		Total	11		
	Tumor size (cm)	Negative	9	13,44	121,00
		Positive	11	8,09	89,00
		Total	20		

Test Statistics ^b		
COX-2 status		Tumor size (cm)
Negative	Mann-Whitney U	8,000
	Wilcoxon W	14,000
	Z	-,816
	Asymp. Sig. (2-tailed)	,414
	Exact Sig. [2*(1-tailed Sig.)]	,497 ^a
Positive	Mann-Whitney U	23,000
	Wilcoxon W	89,000
	Z	-2,022
	Asymp. Sig. (2-tailed)	,043
	Exact Sig. [2*(1-tailed Sig.)]	,046 ^a
a. Not corrected for ties.		
b. Grouping Variable: 4. Rp %		

Case Processing Summary								
COX-2 status		RP status	Cases					
			Valid		Missing		Total	
			N	Percent	N	Percent	N	Percent
Negative	Age (yrs)	Negative	8	100,0%	0	,0%	8	100,0%
		Positive	3	100,0%	0	,0%	3	100,0%
	Tumor size (cm)	Negative	8	100,0%	0	,0%	8	100,0%
		Positive	3	100,0%	0	,0%	3	100,0%
Positive	Age (yrs)	Negative	9	100,0%	0	,0%	9	100,0%
		Positive	11	100,0%	0	,0%	11	100,0%
	Tumor size (cm)	Negative	9	100,0%	0	,0%	9	100,0%
		Positive	11	100,0%	0	,0%	11	100,0%

Descriptives						
COX-2 status		PR status		Statistic	Std. Error	
Negative	Tumor size (cm)	Negative	Mean		4,60	1,040
			95% Confidence Interval for Mean	Lower Bound	2,14	
				Upper Bound	7,06	
			5% Trimmed Mean		4,53	
			Median		4,50	
			Variance		8,649	
			Std. Deviation		2,941	
			Minimum		1	
			Maximum		9	
			Range		8	
		Interquartile Range		6		
		Skewness		,252	,752	
		Kurtosis		-1,641	1,481	
		Positive	Mean		2,20	,208
			95% Confidence Interval for Mean	Lower Bound	1,30	
				Upper Bound	3,10	
			5% Trimmed Mean		.	
			Median		2,30	
			Variance		,130	
			Std. Deviation		,361	

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			Minimum	2		
			Maximum	3		
			Range	1		
			Interquartile Range	.		
			Skewness	-1,152	1,225	
			Kurtosis	.	.	
Positive	Tumor size (cm)	Negative	Mean	4,60	1,355	
			95% Confidence Interval for Mean	Lower Bound	1,47	
				Upper Bound	7,73	
			5% Trimmed Mean	4,17		
			Median	3,80		
			Variance	16,535		
			Std. Deviation	4,066		
			Minimum	2		
			Maximum	15		
			Range	13		
			Interquartile Range	3		
			Skewness	2,563	,717	
		Kurtosis	7,016	1,400		
		Positive	Mean	2,35	,275	
			95% Confidence Interval for Mean	Lower Bound	1,74	
				Upper Bound	2,97	
			5% Trimmed Mean	2,33		
			Median	2,30		
			Variance	,835		
			Std. Deviation	,914		
			Minimum	1		
			Maximum	4		
			Range	3		
			Interquartile Range	1		
Skewness	,797		,661			
Kurtosis	-,346	1,279				

Annex 13

Correlation between Progesterone Receptor Expression and Differentiation Grade according to Cyclooxygenase-2 Expression

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PR status *	31	100,0%	0	,0%	31	100,0%
Differentiation grade *						
COX-2 status						

Crosstab						
COX-2 status				Differentiation grade		Total
				1	2 or 3	
Negative	PR status	Negative	Count	0	8	8
			% within PR status	,0%	100,0%	100,0%
			% within Differentiation grade	,0%	80,0%	72,7%
			% of Total	,0%	72,7%	72,7%
		Positive	Count	1	2	3
			% within PR status	33,3%	66,7%	100,0%
			% within Differentiation grade	100,0%	20,0%	27,3%
			% of Total	9,1%	18,2%	27,3%
	Total	Count	1	10	11	
		% within PR status	9,1%	90,9%	100,0%	
		% within Differentiation grade	100,0%	100,0%	100,0%	
		% of Total	9,1%	90,9%	100,0%	
Positive	PR status	Negative	Count	2	7	9
			% within PR status	22,2%	77,8%	100,0%
			% within Differentiation grade	33,3%	50,0%	45,0%
			% of Total	10,0%	35,0%	45,0%

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		Positive	Count	4	7	11
			% within PR status	36,4%	63,6%	100,0%
			% within Differentiation grade	66,7%	50,0%	55,0%
			% of Total	20,0%	35,0%	55,0%
	Total		Count	6	14	20
			% within PR status	30,0%	70,0%	100,0%
			% within Differentiation grade	100,0%	100,0%	100,0%
			% of Total	30,0%	70,0%	100,0%

Chi-Square Tests						
COX-2 status		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Negative	Pearson Chi-Square	2,933 ^a	1	,087		
	Continuity Correction ^b	,286	1	,592		
	Likelihood Ratio	2,883	1	,090		
	Fisher's Exact Test				,273	,273
	Linear-by-Linear Association	2,667	1	,102		
	N of Valid Cases	11				
Positive	Pearson Chi-Square	,471 ^c	1	,492		
	Continuity Correction ^b	,038	1	,844		
	Likelihood Ratio	,479	1	,489		
	Fisher's Exact Test				,642	,426
	Linear-by-Linear Association	,448	1	,503		
	N of Valid Cases	20				
a. 3 cells (75,0%) have expected count less than 5. The minimum expected count is ,27.						
b. Computed only for a 2x2 table						
c. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 2,70.						

Risk Estimate				
COX-2 status		Value	95% Confidence Interval	
			Lower	Upper
Negative	For cohort Differentiation grade = 2 or 3	1,500	,674	3,339
	N of Valid Cases	11		
Positive	For cohort Differentiation grade = 2 or 3	1,222	,693	2,155
	N of Valid Cases	20		
	Odds Ratio for 4. Rp % (Negativo / Positivo)	,500	,068	3,675
	For cohort Differentiation grade = 1	,611	,143	2,608

Annex 14

Correlation between Progesterone Receptor Expression and Axillary Node Metastasis according to Cyclooxygenase-2 Expression

Case Processing Summary						
	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PR status * Axillary node metastasis* COX-2 status	31	100,0%	0	,0%	31	100,0%

Crosstab						
COX-2 status				Axillary node metastasis		Total
				No	Yes	
Negative	PR status	Negative	Count	4	4	8
			% within PR status	50,0%	50,0%	100,0%
			% within axillary node metastasis	66,7%	80,0%	72,7%
			% of Total	36,4%	36,4%	72,7%
		Positive	Count	2	1	3
			% within PR status	66,7%	33,3%	100,0%
			% within axillary node metastasis	33,3%	20,0%	27,3%
			% of Total	18,2%	9,1%	27,3%
	Total		Count	6	5	11
			% within PR status	54,5%	45,5%	100,0%
			% within axillary node metastasis	100,0%	100,0%	100,0%
			% of Total	54,5%	45,5%	100,0%
Positive	PR status	Negative	Count	5	4	9
			% within PR status	55,6%	44,4%	100,0%
			% within axillary node metastasis	31,3%	100,0%	45,0%
			% of Total	25,0%	20,0%	45,0%

		Positive	Count	11	0	11
			% within PR status	100,0%	,0%	100,0%
			% within axillary node metastasis	68,8%	,0%	55,0%
			% of Total	55,0%	,0%	55,0%
	Total		Count	16	4	20
			% within PR status	80,0%	20,0%	100,0%
			% within axillary node metastasis	100,0%	100,0%	100,0%
			% of Total	80,0%	20,0%	100,0%

Chi-Square Tests						
COX-2 status		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Negative	Pearson Chi-Square	,244 ^a	1	,621		
	Continuity Correction ^b	,000	1	1,000		
	Likelihood Ratio	,249	1	,618		
	Fisher's Exact Test				1,000	,576
	Linear-by-Linear Association	,222	1	,637		
	N of Valid Cases	11				
Positive	Pearson Chi-Square	6,111 ^c	1	,013		
	Continuity Correction ^b	3,649	1	,056		
	Likelihood Ratio	7,651	1	,006		
	Fisher's Exact Test				,026	,026
	Linear-by-Linear Association	5,806	1	,016		
	N of Valid Cases	20				
a. 4 cells (100,0%) have expected count less than 5. The minimum expected count is 1,36.						
b. Computed only for a 2x2 table						
c. 2 cells (50,0%) have expected count less than 5. The minimum expected count is 1,80.						

Risk Estimate				
COX-2 status		Value	95% Confidence Interval	
			Lower	Upper
Negative	Odds Ratio for PR status (Negative / Positive)	,500	,031	7,994
	For cohort Ganglion metastasis = No	,750	,260	2,162
	For cohort Ganglion metastasis = Yes	1,500	,262	8,579
	N of Valid Cases	11		
Positive	For cohort Ganglion metastasis = No	,556	,310	,997
	N of Valid Cases	20		

Annex 15

Journal Article Submitted for Publication

Cyclooxygenase-2 immunoexpression in breast cancer: Progesterone Receptors Influence

Micaela Almeida, Javier Muñoz, José Moutinho

Abstract

In breast cancer Cyclooxygenase-2 overexpression is related with poor outcomes, but the clinical relevance of Cyclooxygenase-2 is still unclear. Progesterone Receptor is a regulator of Cyclooxygenase-2 expression. We postulate that Progesterone Receptor was associated to Cyclooxygenase-2 expression and clinicopathologic factors in breast cancer. Cyclooxygenase-2 and Progesterone Receptor expression was analyzed, by immunohistochemistry, in 31 cases of invasive ductal carcinoma. The expression of Cyclooxygenase-2 and progesterone receptor was observed in 64.5% and 45.2% of the tumors, respectively. We found tumors 11 with both Cyclooxygenase-2 and progesterone receptor overexpression and they are related with tumor lower size and lower axillary node metastasis. These results suggest that progesterone receptor has a protective role in breast cancer by inhibiting chronic inflammatory pathway.

Introduction

Cyclooxygenase-2 (COX-2) is the induced cyclooxygenase (COX) isoform, is expressed in most tissues and commonly expressed in more than 50% of invasive breast cancers [1, 2]. COX-2 is involved in chronic inflammation and is responsible for arachidonic acid (AA) conversion into prostaglandins, which are associated with increased aromatase that in turn results in high local estrogen receptor (ER) levels [3-7].

It has long been thought that ER induced progesterone receptor (PR) and that serves as an indicator of ER functional capacity; new studies indicate that PR is an independent prognostic risk factor predictor and may have protective actions by antagonizing inflammatory response pathway (fig. 1) [3].

PR impairs transactivation of COX-2 through nuclear factor kappa B (NF- κ B) via inhibitory factor κ B α (I κ B α) [8, 9]. NF- κ B inhibition by PR, results in estrogen lower levels. In that way and PR modulates breast tumor development and progression [3, 10].

In clinical ground PR has protective role in breast cancer, this is the reason why ER⁺/PR⁻ tumor subtype are more aggressive, have high proliferation rates and higher recurrence than ER⁺/PR⁺ [8].

The aim of this study was to determine if COX-2 and PR immunoexpression, had correlation with clinicopathologic factors (age, tumor size, histologic grade and axillary node metastasis), which are known to be prognostic factors.

Methods

Patient selection

There were selected 31 patients who were diagnosed, between 2007 and 2009, with breast cancer in Child and Women department of Cova da Beira Medical Center in Portugal. All cases were invasive ductal carcinoma. There were excluded tumors with less than 10 mm, to preserve archive material. There were excluded all pregnant women and patients submitted to primary chemotherapy or hormonotherapy. In all cases there was no distant metastasis, all patients were submitted to radical breast tumor excision and sentinel mapping lymphadenectomy. Although the short period of time there was not recorded any case of recurrence.

Immunostaining

Paraffin-embedded samples of 3 μ m were cut and processed for immunostaining. For each case of study were selected three slides, one for hematoxylin-eosin staining and the remaining two for COX-2 and PR immunostaining. There was performed a dewaxing with xylene, followed by rehydration in decreasing ethanol grades and water. For antigen retrieval, slides were put into pressure cooker and citrate buffer 0.01 M for 6 minutes at pH 6 and washed with water and buffer solution (K5006, ChemMat™, Dako). Slides were incubated with the primary antibody, for COX-2 staining was used clone SP21, rabbit

monoclonal antibody (MC-16-240r, CellMarque™) at 1:10 dilution; for PR staining were used clone 16 and clone SAN27 (NCL-L-PGR-AB, Novocastra™) at 1:100 dilution, in both cases the diluent used was S2022 (Dako Real). Incubation with the primary anti-body was made for 25 minutes at room temperature, then washed with buffer solution (K5006, ChemMat™, Dako). Incubation with biotinylated secondary antibody was made for 25 minutes, using the bottle A from K5001 kit (Dako Real™), followed by washing with buffer solution (K5006, ChemMat™, Dako). To block endogenous peroxidase there was used a blocking-Peroxidase solution (S2023, Dako Real™) for 7 minutes and 30 seconds, followed by washing with buffer solution (K5006, ChemMat™, Dako). It was added streptavidin conjugated horseradish peroxidase (HRP) (bottle B, K5001, Dako Real), for 25 minutes followed by washing with buffer solution (K5006, ChemMat™, Dako), chromogen was added (Dako Real DAB + chromogen, bottle C + HRP substrate buffer bottle D, K5001, Dako Real) at a 20:1000 dilution for 15 min with washes at every 5 minutes, this was followed by washing with a buffer solution (K5006, ChemMat™, Dako). The counterstaining was done with Mayer's hematoxylin for 1 min and washed with a buffer solution (K5006, ChemMat™, Dako), dehydrated in increasing ethanol grades (75%, 90%, absolute) and xylene. Slides were preserved in synthetic mounting medium, and were analyzed with an optical microscope (x400), using 50 fields for slide.

For Cox-2 was considered cytoplasmatic immunostaining and to PR was recorded nuclear immunostaining.

The Immunostaining intensity was recorded, by an independent pathologist, as: negative if no staining; high if staining is similar to positive control and Moderate if intensity of the staining is less than positive control.

Positivity of Immunostaining was recorded as percentage of the cells that stained, independently of intensity of the staining. It was counted all cells in an optical high power field considered representative of all slide, in a minimum of 500 cells. For statically analysis we considered staining in less than 25% of the cell as a negative result; 25 to 50% of the cells as light expression; 50-75% as moderate expression and 75 to 100% as a high expression.

For comparative statically analysis we considered as positive cases immunostaining in at least 50% of the cells and moderate and high intensity of the staining. All other was considered as negative cases.

Statistical analysis

Statistical analysis was performed using SPSS, version 17. Mann-Whitney and Chi-Squared tests were used for the analysis, considering a statistical significance when P-value was < 0.05.

Results

The analyzed clinical cases were between the year 2007 and 2009 and the age of patients varied from 39 to 91 years old. Most of the tumors expressed COX-2 (64.52%) and 45.16% of the tumors expressed PR. It was also taking into account the differentiation grade and axillary node metastasis, and we verified that twenty four of tumors had a high (G2 or G3) differentiation grade and only 29.03% of women had axillary node metastasis (table 1).

COX-2 expression was considered positive by semiquantitative scoring in 20 of 31 cases studied (64,52%), all 20 cases had a moderate or high expression (equal or greater than 50%). Immunostaining intensity was considered moderate in 18 cases and high in 2 cases (fig. 2). PR expression was detected in 14 of 31 cases in study (45,16%), all 14 cases had moderate or high expression (equal or greater than 50%) (fig. 3).

Correlation of COX-2 and PR expression with clinicopathological parameter

In order to evaluate any association between PR and COX-2 immunoexpression it was constituted 4 groups: positive COX-2 expression with positive PR expression (COX-2⁺/PR⁺), positive COX-2 expression with negative PR expression (COX-2⁺/PR⁻), negative COX-2 expression with positive PR expression (COX-2⁻/PR⁺) and negative COX-2 expression with negative PR expression (COX-2⁻/PR⁻).

COX-2⁺/PR⁺ group is constituted for 11 of 31 cases (35.48%), COX-2⁺/PR⁻ group is constituted for 9 of 31 cases (29.03%), COX-2⁻/PR⁺ group is constituted for 3 of 31 cases (9.68%) and COX-2⁻/PR⁻ group is constituted for 8 of 31 cases (25.81%) (table 2). These groups were correlated with clinicopathological factors and we found that COX-2⁻/PR⁻ group is more heterogeneous, ranging the size from 14mm to 90mm, and the group COX-2⁻/PR⁺ the more homogeneous (fig. 4a), however there was not found statistical significance (p = 0.414). Similar results were found for COX-2⁺/PR⁻ group, with higher standard deviation (4.066) and higher mean (4.60) than COX-2⁺/PR⁺ group, which as 0.914 and 2.35 for standard deviation and mean, respectively (fig. 4b). Comparing these two groups we found statistical significance (p < 0.05).

Comparing COX-2 expression and PR expression with axillary node metastasis we found that COX-2⁻/PR⁻ group has a higher number of cases with axillary node metastasis (n = 4) and without axillary node metastasis (n = 4) than COX-2⁻/PR⁺ group (fig. 5a), but there was not found statistical significance. Unlike these results, we found statistical significance between COX-2⁻/PR⁻ and COX-2⁺/PR⁺ groups (p < 0.05). In COX-2⁺/PR⁺ there are no cases with axillary node metastasis and in COX-2⁻/PR⁺ group there are 4 cases (44.4%) with axillary node metastasis and 5 cases (55.6%) without axillary node metastasis (fig.5b).

There was also performed a correlation between COX-2 and PR expression with age and differentiation grade, however there was not found statistical significance in any case (data not shown).

Discussion

This study evaluates COX-2 and PR immunostaining only in ductal invasive breast cancer in patients not submitted to primary chemotherapy, radiotherapy or hormone therapy. In our knowledge is the first study that evaluates specifically that kind breast cancer. There was studied only invasive ductal tumors because they are more frequent and their behavior was more clarified. The role of chronic inflammation in breast cancer is poorly understood. COX-2 overexpression is a marker of chronic inflammation, PR has been appointed as a modulator of COX-2. Our study suggests that COX-2 and PR expression are related with lower size and lower tumor node metastasis. These results have no concordance with that ones found by Lee et al. who did not establish a correlation between PR expression, COX-2 expression and clinicopathological factors [11]. The discrepancy on their results may be reflecting different methodology: they considered >10% PR or COX-2 of staining as a positive case and in our study only the cases with at least 50% of staining were considered as positive. Our methodology seems more reasonable in order to avoid false positive cases. Age and tumor differentiation grade represents important factors for breast cancer, but in our study COX-2 and PR immunostaining had not significant statistical association with clinical and pathological factors, probably due to the low number of cases studied.

Despite PR role in breast cancer is unclear, Hardy et al. verified in cellular breast cancer lines, PR antagonizes COX-2 expression [10]. PR acts as a dependent and independent ligand to inhibit NF- κ B (COX-2 transcriptional factor) transactivation [3, 10]. PR interferes with COX-2 transactivation through NF- κ B, via I κ B α induction, which modulates COX-2 expression. COX-2 expression augments aromatase activity with consequent local increase of ER [3, 10]. PR decreases COX-2 expression, with consequent decrease of activity and ER levels [3, 10]. Probably this is the reason why PR⁻ hormone tumors are more aggressive and have higher proliferation rates when compared with PR⁺ hormone tumors, in a independent way of ER immunostaining [8]. Thus, high levels of PR in breast epithelial cells, plays a protective role in breast cancer, by modulating inflammatory response pathway.

Our results have shown that COX-2⁺/PR⁺ was associated with lower tumor size and lower axillary node metastasis, suggesting that PR positive ductal invasive breast cancer may have different behavior in according of COX-2 expression. In fact, COX-2⁺/PR⁺ immunoexpression seems to represent a marker for better prognosis. We speculate if COX-2 expression will be related with better disease-free survival and overall-survival in patients immunopositive for PR submitted to adjuvant hormonal therapy. Clinical studies may clarify the usefulness of the COX-2 immunostaining in clinical practice.

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Images

Table 1: Patient characteristics: Year, Age, COX-2 expression, PR expression, Tumor Size, Differentiation grade and axillary node metastasis.

Characteristics	N.º of Patients	(%)
Year:		
2007	13	41.94%
2008	15	48.39%
2009	6	19.35%
Age:		
< 40	2	6.45%
≥40 <50	2	6.45%
≥50 <60	3	9.68%
≥60 <70	6	19.35%
≥70 <80	9	29.03%
≥80 <90	8	25.81%
≥90	1	3.23%
COX-2 staining:		
Negative	11	35.48%
Moderate	18	58.06%
High	2	6.45%
COX-2 expression:		
0-25%	11	35.48%
25%-50%	14	45.16%
50%-75%	5	16.13%
75%-100%	1	3.23%
PR staining:		
Negative	17	54.84%
Moderate	10	32.26%
High	4	12.90%
PR expression:		
0-25%	17	54.84%
25%-50%	5	16.13%
50%-75%	6	19.35%
75%-100%	3	9.68%
Tumor size (mm):		
< 20	10	32.26%
≥20 <50	15	48.39%
≥50	6	19.35%
Differentiation grade:		
G1	7	22.58%
G2	16	51.61%
G3	8	25.81%
Axillary node metastasis:		
Present	9	29.03%
Absent	22	70.97%

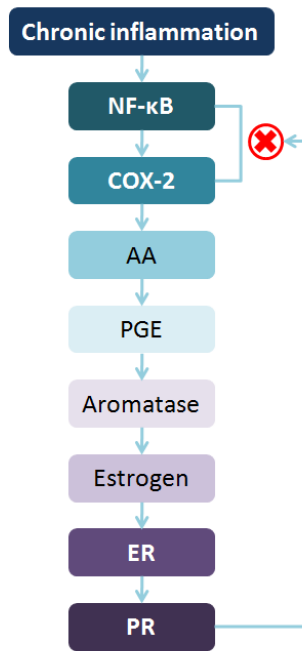


Figure 1: COX-2 is expressed in chronic inflammation, converting AA into PGE, leading to aromatase increase with subsequent increase of ER and PR. PR through NF-κB results in COX-2 lower expression.

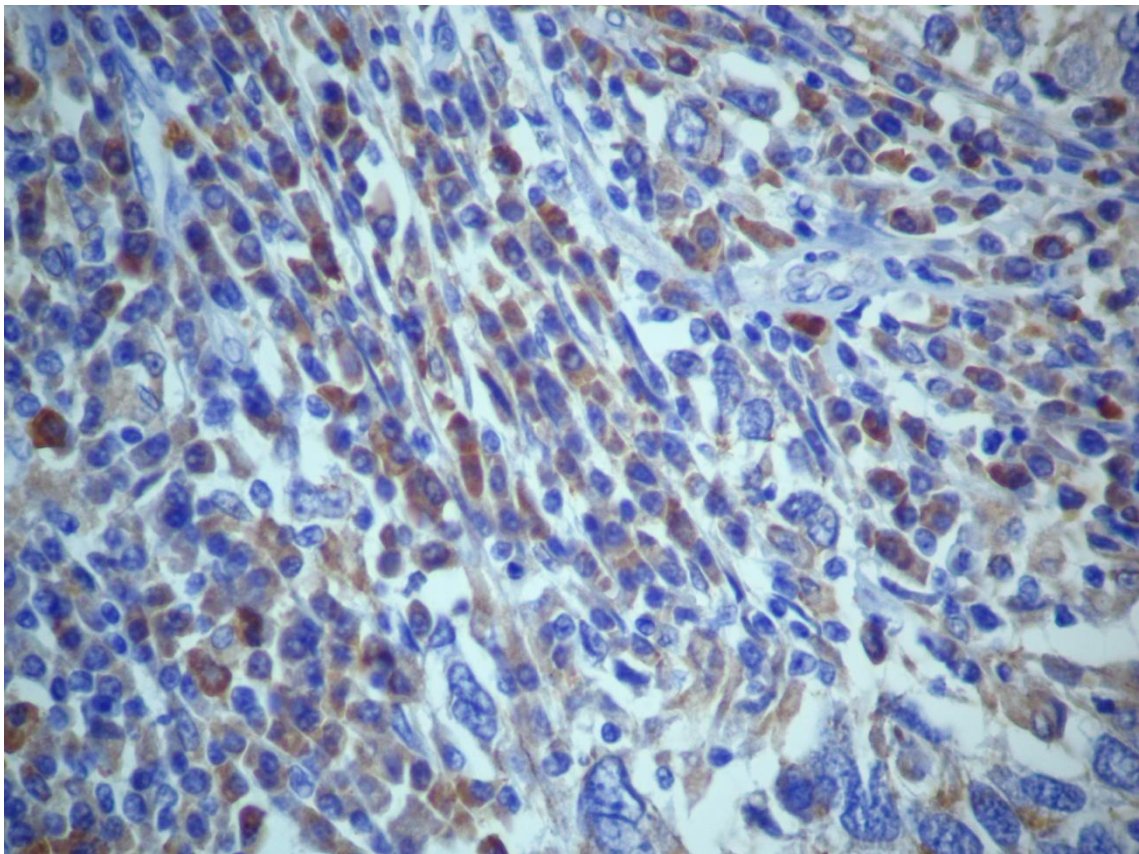


Figure 2: Immunohistochemical localization of COX-2 in ductal carcinoma (400x magnification). There is cytoplasmic staining with COX-2 light expression.

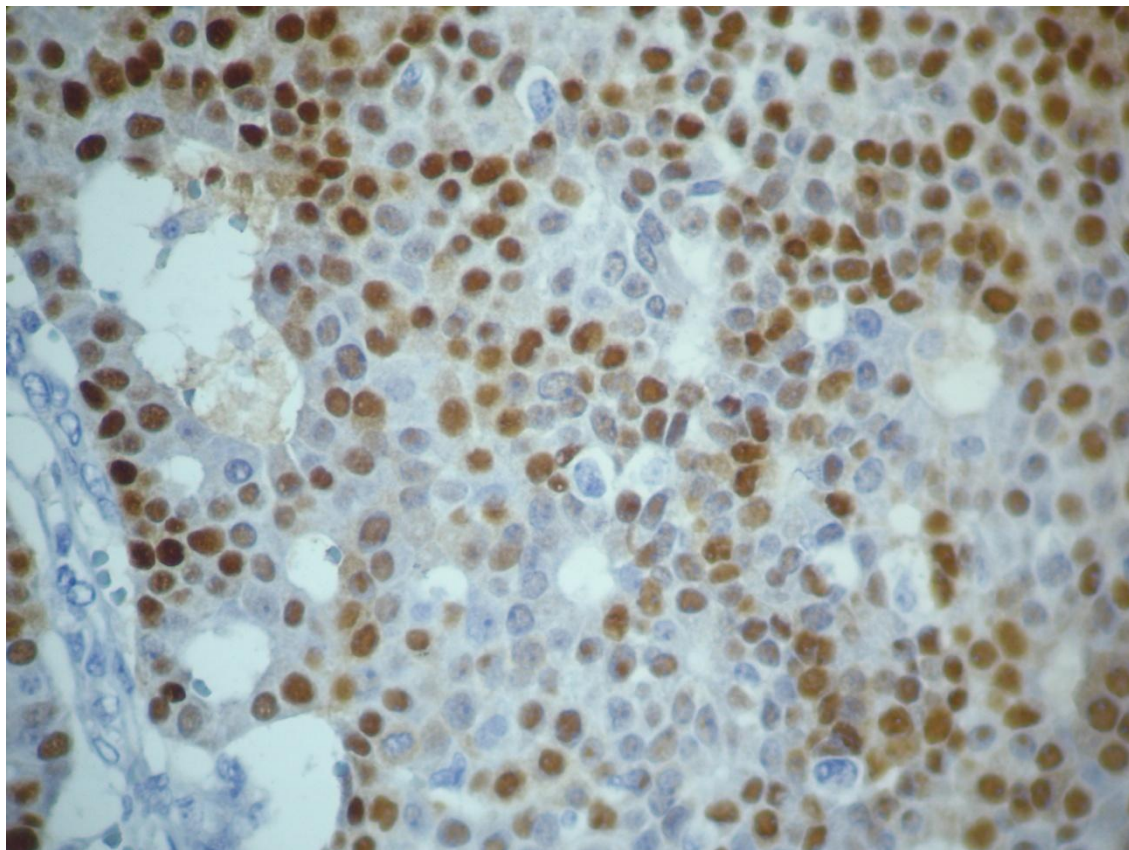


Figure 3: Immunohistochemical localization of PR in ductal carcinoma (400x magnification). There is nuclear staining with PR light expression.

Table 2: Correlation between COX-2 and PR expression.

	COX-2 expression	COX-2 expression	Total
PR positive status	n = 11 (35.48%)	n = 3 (9.68%)	14 (45.16%)
PR negative status	n = 9 (29.03%)	n = 8 (25.81%)	17 (54.84%)
Total	20 (64.51%)	11 (35.49%)	31 (100%)

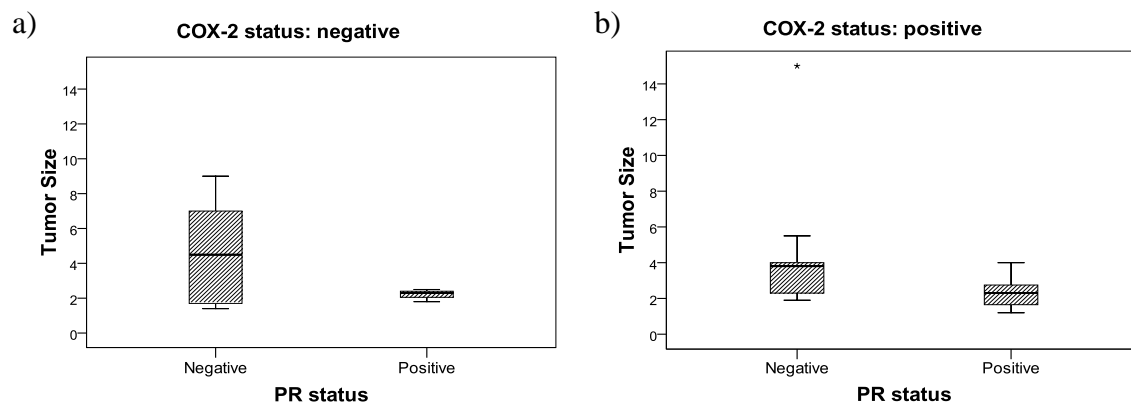


Figure 4: Correlation between PR expression and tumor size according to COX-2 expression. a) The COX-2⁻/PR⁻ group is more heterogeneous and has higher mean than COX-2⁻/PR⁺ group. b) COX-2⁺/PR⁺ group has a lower mean and standard deviation than COX-2⁺/PR⁻ group.

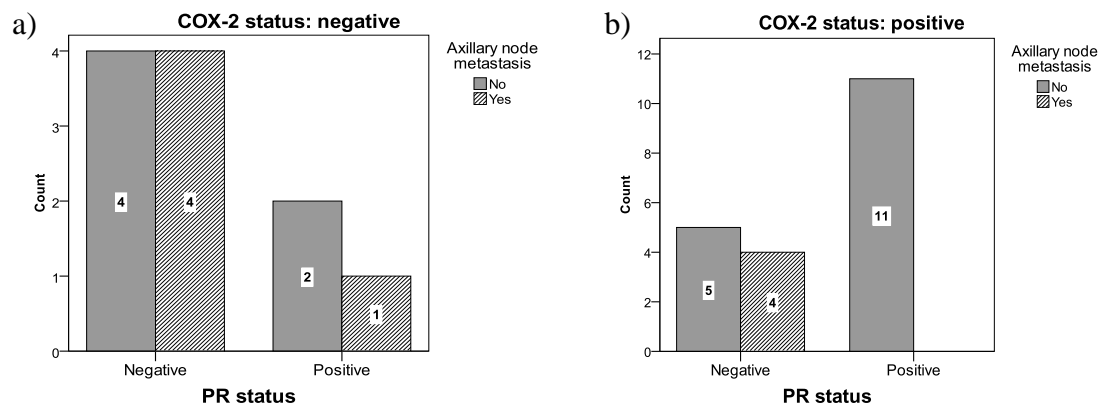


Figure 5: Correlation between PR expression and axillary node metastasis according to COX-2 expression. a) In COX-2⁻/PR⁻ there is the same number of cases with and without axillary node metastasis, for PR status negative there are more cases without axillary node metastasis. b) COX-2⁺/PR⁻ has a higher number of cases with axillary node metastasis than COX-2⁺/PR⁺ group, which has only cases without axillary node metastasis.