Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

Expression of aquaporins in pancreatic tumors: new targets for cancer therapy?

Inês Gomes Direito

Dissertação orientada pela Prof.ª Doutora Graça Soveral
e pelo Prof. Doutor Manuel Carmo Gomes

Mestrado em Biologia Molecular e Genética

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“A mente que se abre a uma nova ideia,

jamais volta ao seu tamanho inicial”

ALBERT EINSTEIN

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Abstract

Background: Aquaporins (AQPs) belong to a group of membrane proteins involved in the transport of water and small solutes such as glycerol, through the plasma membranes of many tissues and cell types. The 13 AQPs found in mammals are present in many epithelial, endothelial and other tissues. Interestingly, some isoforms such as aquaporin-5 (AQP5), proved to be highly expressed in different tumor types having a role in tumorigenesis. In view of the wide range of cancer malignancies in which AQP5 has been detected, an increasing interest in its implication in carcinogenesis has emerged. Recent publications suggest that AQP5 is up-regulated in a variety of systemic malignancies being related with tumor recurrence, metastasis and poorer patients prognosis by enhancing cancer cell proliferation, migration and survival.

Pancreatic cancer is the fourth most common cause of cancer deaths worldwide and has a 5 year survival rate of <5%. The high mortality rates can, in part, be attributed to diagnosis only at advanced stage. The disclosure of specific prognosis biomarkers enabling to predict the outcome of the patient after surgery and thus to establish adequate therapeutics is therefore desirable. In normal pancreatic tissue AQP5 is expressed at the apical membrane of intercalated and intralobular pancreatic ductal cells, playing an important role in transcellular water flux for final isotonic pancreatic fluid production. Despite AQP5 physiological role in pancreas, little is known about its expression and roles in pancreatic ductal adenocarcinoma (PDA).

The aim of the present study was to investigate the expression pattern of AQP5 in PDA and its clinical significance.

Methods: To evaluate the potential of AQP5 as a novel biomarker for PDA, we performed immunohistochemical studies in 35 human PDA and in correspondent normal pancreatic tissues (control samples). Statistical analysis was also performed to investigate associations between AQP5 expression and the expression of a proliferative cell marker (Ki-67), a PDA marker (Cytokeratin 7), an epithelial cell marker (E-cadherin) and a mesenchymal cell marker (Vimentin).

Results: AQP5, Ki-67, CK7 and Vim are overexpressed in PDA samples while E-cad protein levels are down-regulated when compared with control samples. AQP5 and E-cad expression were found to be related with moderately histological differentiation while Ki-67 expression was correlated with tumor location and Vim expression. AQP5 and CK7 were also detected in some scattered cells in duodenum that do not express these proteins in normal conditions suggesting the presence of infiltrative tumor cells.

Conclusions: AQP5 is up-regulated in PDA and is associated with a loss of polarity in ductal epithelial cells and with tumor differentiation/ grade. Our results suggest that AQP5 might be

used as a biomarker for PDA aggressiveness allowing the detection of early disease stages. Due to its putative role in PDA tumorigenesis AQP5 may also represent a potential drug target for this disease.

Keywords: Aquaporin-5, cancer, tumor, biomarker, pancreas

Resumo

Introdução: As aquaporinas (AQPs) pertencem a um grupo de proteínas membranares envolvidas no transporte de água e pequenos solutos, tais como glicerol, através das membranas plasmáticas de diferentes tipos celulares, desempenhando um papel fundamental na homeostasia da água que é crucial para todas as células vivas. Nos mamíferos foram já identificadas 13 AQPs, presentes nos mamíferos, estando estas 13 isoformas amplamente distribuídas pelos diversos tecidos do corpo humano, tais como epitélios, endotélios e outros. Curiosamente, algumas destas isoformas são altamente expressas em diferentes tipos de tumores, desempenhando um papel relevante na tumorigénese. Uma destas isoformas, a Aquaporina-5 (AQP5), tem vindo a ser detetada numa grande variedade de tumores, pelo que o interesse no estudo das suas possíveis implicações na carcinogénese tem vindo a crescer nos últimos anos. Publicações recentes sugerem que a AQP5 está relacionada com a recorrência da doença, com o aparecimento de metástases e, consequentemente, um pior prognóstico para os doentes. A sua sobre-expressão tem vindo ainda a ser implicada na promoção da proliferação das células tumorais, na sua capacidade de migração e sobrevivência. Apesar de não serem ainda totalmente conhecidos os mecanismos pelos quais esta proteína participa na tumorigénese, sabe-se que a AQP5 poderá interagir com oncoproteínas, tais como RAS e c-Src. A interação com oncoproteínas promove a sinalização intracelular, a qual poderá resultar na proliferação das células tumorais, na sua sobrevivência, no aumento da sua capacidade de migração bem como no aumento da sua resistência a fármacos.

No tecido pancreático normal a AQP5 é expressa na membrana apical das células dos ductos intercalares e intralobulares, onde desempenha uma importante função no fluxo transcelular de água necessário à produção do fluido pancreático final com características isotónicas. Apesar do papel fisiológico que esta isoforma desempenha no tecido pancreático normal, pouco se sabe acerca da sua expressão e possíveis funções no Adenocarcinoma ductal do Pâncreas (PDA). A nível mundial, o cancro do pâncreas é a quarta causa mais comum de mortes causadas por cancro, tendo uma taxa de sobrevivência a 5 anos inferior a 5%. As altas taxas de mortalidade podem, em parte, ser atribuídas ao diagnóstico tardio, já em fases avançadas da doença (cerca de 50% dos doentes apresenta focos metastáticos distantes aquando do diagnóstico). Devido à localização retroperitoneal do pâncreas os estadios precoces deste tipo de cancro normalmente não produzem sintomas. A descoberta de biomarcadores específicos que permitam prever o prognóstico do paciente após cirurgia e estabelecer uma terapêutica adequada é, portanto, necessária. Assim sendo, o principal objetivo do presente estudo foi investigar o padrão de expressão da AQP5 e a sua significância clínica no PDA.

Metodologia: Foram realizados testes imunohistoquímicos para determinar a expressão de AQP5, Ki-67 (marcador de proliferação celular), Citoqueratina 7 (CK7) (marcador de PDA), E-caderina (E-cad) (marcador de células epiteliais) e Vimentina (Vim) (marcador de células mesenquimais) em 35 amostras de PDA (com diagnóstico histológico bem estabelecido) e respetivos tecidos pancreáticos normais (controlo para fins comparativos) recolhidas de pacientes submetidos a ressecção cirúrgica. As amostras foram avaliadas ao microscópio consoante os seguintes critérios: intensidade da marcação imunohistoquímica (0, sem marcação; 1, marcação fraca; 2, marcação moderada; 3, marcação forte) e percentagem de células marcadas (0, <10% células marcadas; 1, ≥10% <50% células marcadas e 2, ≥50% células marcadas. A expressão de cada marcador foi definida como um *score* imunohistoquímico final como resultado da soma dos dois critérios avaliados. Os marcadores foram considerados sobreexpressos nas amostras de PDA quando o *score* imunohistoquímico final foi superior ao valor médio do score imunohistoquímico final nas amostras controlo. Testes estatísticos relevantes foram aplicados para o estabelecimento de correlações entre os marcadores analisados e as variáveis clinicas dos doentes.

Resultados e discussão: Verificou-se que nas amostras de PDA os marcadores AQP5, Ki-67, CK7 e Vim estão sobreexpressos, ao passo que a E-cad se encontra subexpressa, por comparação com as amostras controlo.

Verificou-se que a expressão de Ki-67 se relaciona com a localização do tumor (cabeça do pâncreas) e com a expressão de Vim. Por sua vez, a expressão de AQP5, bem como a expressão de E-cad relacionam-se com a diferenciação histológica do tumor, sendo os níveis de expressão mais elevados encontrados em tumores moderadamente diferenciados. Sabe-se que os tumores moderadamente diferenciados recapitulam melhor as características morfológicas dos ductos do pâncreas normal do que os tumores pouco diferenciados, estes últimos com uma morfologia mais desagregada, menos coesa, o que está de acordo com os níveis de E-cad mais reduzidos. Deste modo, e apesar de serem necessários estudos adicionais, estas observações preliminares sugerem que a AQP5 poderá ser utilizada como um biomarcador para os estadios mais precoces da doença e que a sua expressão poderá estar relacionada com um melhor prognóstico para os doentes, uma vez que os tumores moderadamente diferenciados apresentam um comportamento menos agressivo do que os tumores pouco diferenciados.

Observou-se uma perda de polaridade na expressão de AQP5 nas células das amostras de PDA: esta deixou de estar apenas expressa nas membranas apicais das células dos ductos intercalares e intralobulares do pâncreas passando a expressar-se em toda a membrana plasmática e mesmo intracelularmente. Uma vez que a tumorigénese ocorre nas células epiteliais ductais estes achados sugerem que a AQP5 poderá estar envolvida na progressão do PDA, podendo representar um potencial *drug target* para esta doença.

Observou-se ainda a marcação de AQP5 e de CK7 em algumas células dispersas no epitélio duodenal, em casos com infiltração intestinal. Uma vez que em condições normais as células epiteliais do duodeno não expressam nenhuma destas proteínas, estes resultados indicam a presença de células tumorais infiltrativas. É ainda importante salientar que se observou uma imunomarcação mais forte destas células com AQP5 do que com CK7, o que sugere que a utilização de AQP5 como biomarcador para deteção de células tumorais infiltrativas poderá ser mais sensível que a sua deteção com CK7.

Conclusões e perspetivas: A sobreexpressão de AQP5 tem sido extensamente reportada em diversos tipos de tumores. Observações consistentes sugerem uma forte implicação desta isoforma na carcinogénese em diferentes órgãos e sistemas. Neste estudo, é verificado pela primeira vez, que a AQP5 se encontra sobreexpressa no PDA, associada a uma perda da polaridade na membrana apical das células ductais. A sobreexpressão de AQP5 está ainda associada à diferenciação histológica do tumor.

Devido ao involvimento da AQP5 na migração, proliferação e adesão celular em cancros humanos, esta proteína surge com um alvo farmacológico promissor e os seus moduladores como agentes ati-tumorais vantajosos. Apesar dos mecanismos pelos quais a AQP5 participa na tumorigénese ainda não serem completamente conhecidos, a informação disponível suporta a sua interação com ocoproteínas e o seu envolvimento em cascatas intracelulares de transdução de sinal. A sua função como canal, responsável pelo transporte de água ou de outra qualquer molécula sinalizadora poderá também ser crucial para a tumorigénese. A complexidade do uso terapêutico da AQP5 é ainda ilustrada pelo seu envolvimento em mecanismos tumorais de sensibilidade e resistência a fármacos.

Em conclusão, existem diversas evidências que suportam a potencial aplicação da AQP5 como alvo terapêutico e como ferramenta de diagnóstico. Tendo em conta os inúmeros tipos de cancro em que esta proteína se encontra envolvida a potencial utilização da AQP5 como biomarcador na deteção de tumores e no estabelecimento de diagnósticos e prognósticos deverá continua a ser explorada. Os nossos resultados sugerem que esta isoforma poderá ser um promissor biomarcador no que concerne à agressividade do PDA, permitindo a deteção de estadios precoces da doença. Devido ao seu aparente papel na tumorigénese do PDA a AQP5 poderá também representar um potencial alvo terapêutico para esta doença. No entanto, é ainda necessária investigação e validação para que a AQP5 seja aceite como biomarcador para a deteção e seguimento do PDA.

Palavras-chave: Aquaporina-5, cancro, tumor, biomarcador, pâncreas

Abbreviations

α-SMA, Smooth muscle actin α;
ACC, Adenoid cystic carcinoma;
AQP, Aquaporin;
CDK, Cyclin-dependent kinases;
CHIP28, channel-like integral protein of 28 kDa;
CHO, Chinese hamster ovary;
CK7, Cytokeratin 7;
CML, Chronic myelogenous leukemia;
CRC, Colorectal cancer;
E-cad, E-cadherin;
EGCG, Epigallocatechin gallate;
EGFR, Epidermal growth factor receptor;
EMT, Epithelial mesenchymal transition;
ERK1/2, Extracellular signal-regulated kinases 1 and 2;
FAK, Focal Adhesion Kinase;
FIS, Final immunohistochemistry score;
HCC, Hepatocellular carcinoma;
HER2, Epidermal growth factor receptor 2;
IkBα, Nuclear factor-kB inhibitor alpha;
MAPK, Mitogen-activated protein kinases;
MIP, major intrinsic protein;
MUC5AC, Mucin 5AC;
MUC5B, Mucin 5B;
MDR, Multidrug resistance;
NF-kB, Nuclear factor-kB;
NPA, Asparagine-proline-alanine;
NSCLC, Non-small cell lung cancer;
PC, Pancreatic Cancer;
PCNA, Proliferating cell nuclear antigen;
PDTC, Ammonium pyrrolidine dithiocarbamate;
PDA, Pancreatic Ductal Adenocarcinoma;
PKA, Protein kinase A;
PPC, Percentage of positive cells;
pTNM, Pathological tumor/node/metastasis;
Rb, Retinoblastoma protein;
SI, Staining intensity;
SSC, Squamous cell carcinoma;
Vim, Vimentin; ;
ZO-1, Zonula accludens-1

1. Introduction

1.1 Aquaporins

Water homeostasis is central to the physiology of all living cells. Channels that facilitate water permeation through cell membranes, nowadays known as aquaporins (AQPs) were first described on red blood cells in the late 1950s [2] and later on renal epithelia [3]. The first recognized water channel, initially designated as CHIP28 (channel-like integral protein of 28 kDa) [4], was accidently isolated and co-purified with the Rh blood group antigen from erythrocytes membranes by Agre and co-workers in 1987 [5]. The deduced protein sequence [6] revealed a shared homology with the major intrinsic protein (MIP) from bovine lens cells suggesting that this protein belongs to the MIP family of transmembrane channel proteins [4]. Further experiments with CHIP28 inserted in liposomes [7] and heterologously expressed in *Xenopus oocytes* [8], revealed its water channel activity and finally unveiled the protein entity predicted 30 years before. In 2003 Agre and co-workers were awarded the Nobel Prize in Chemistry for the identification and characterization of CHIP28 that was later renamed aquaporin-1 (AQP1). Ever since the discovery of AQP1, many other membrane proteins of the MIP family have been found, today comprising more than 1700 integral membrane proteins present in virtually all-living organisms [9]. In mammals 13 AQPs were identified with specific organ, tissue, and cellular localizations. Importantly, these proteins are involved in many biological functions including transepithelial fluid transport [10], brain edema, neuroexcitability [11], cell migration [12], adhesion [13], proliferation [14, 15], differentiation [16] and metabolism [17].

The most remarkable feature of AQP channels is their high selectivity and efficiency on water or glycerol permeation. AQPs allow water/glycerol to move freely and bidirectionally across the cell membrane in response to osmotic and/or hydrostatic gradients, but exclude ions, such as hydroxide, hydronium ions and protons [18], the latter being essential to preserve the electrochemical potential across the membrane. Apart from water and glycerol, many other permeants such as urea [19], hydrogen peroxide [20], ammonia [21], nitrate [22], arsenite/antimonite [23, 24], nitric oxide [25], silicon [26], carbon dioxide [27] and even anions [28] permeate specific AQPs.

Based on their primary sequences (Figure 1), mammalian AQPs are divided in three subfamilies: classical or orthodox AQPs, aquaglyceroporins and unorthodox AQPs [29]. This subdivision also reflects AQPs permeation specificities: i) orthodox or classical AQPs (AQP0, 1, 2, 4, 5, 6, 8) are primarily selective to water, ii) aquaglyceroporins (AQP3, 7, 9, 10) are permeable to water and small neutral solutes such as glycerol, and iii) unorthodox AQPs (AQP11, 12), also named “S-aquaporins”, ‘‘superaquaporins’’, ‘‘subcellular aquaporins’’ or

‘‘sip-like aquaporins” are located intracellularly with selectivity not clearly established [30, 31], although recent reports revealed AQP11 ability for water [32] and glycerol [33] permeation.

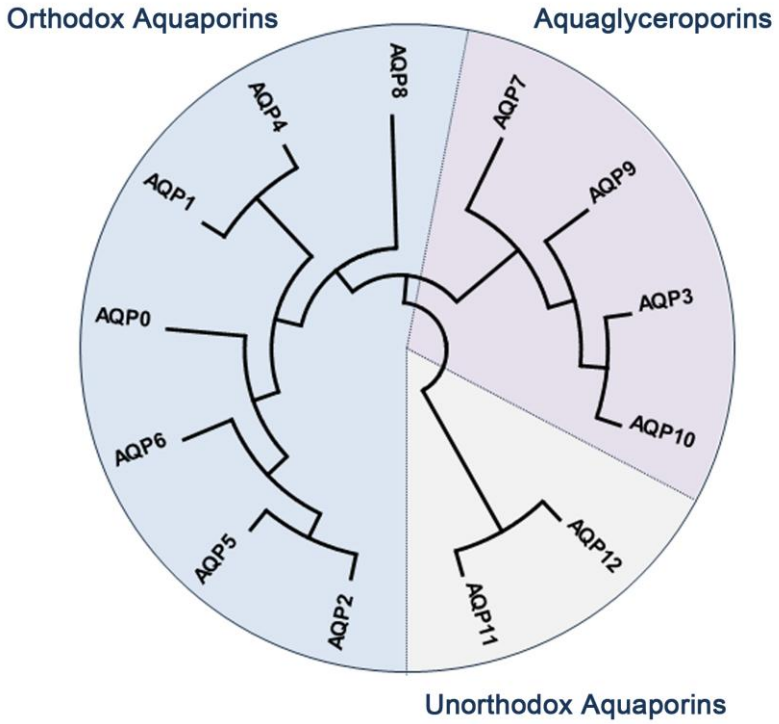


Figure 1 - Dendrogram depicting the phylogenetic relationship of mammalian aquaporins (AQP), showing the three main subfamilies. Dendogram was generated by neighbour-joining method (applied to 1000 bootstrap data sets) using the MEGA6 software [34]. The accession numbers are as follows: MIP (XP_011536656), AQP1 (NP_932766), AQP2 (NP_000477), AQP3 (NP_004916), AQP4 (NP_001641), AQP5 (NP_001642), AQP6 (NP_001643), AQP7 (NP_001161), AQP8 (NP_001160), AQP9 (NP_066190), AQP10 (NP_536354), AQP11 (NP_766627), AQP12 (NP_945349).

Recently, a crescent interest has been paid to AQPs and their roles in cancer development. It is well established that tumor growth, development, invasion and metastasis depend on tumor microenvironment and metabolism [35] and it is also known that AQPs play an important role in tissue water balance in response to osmotic gradients, essential to maintain cell function, including in malignant cells [1, 36]. AQPs may also facilitate tumor growth, local infiltration and metastasis by enhancing cell migration and angiogenesis towards a chemotactic stimulus. The exact mechanism is not clear yet, but may involve rapid changes in cell shape and volume induced by AQPs polarization at the front edge of migrating cells, facilitating transmembrane water fluxes driven by changes in osmolality (produced by transmembrane ion flux) that promote lamellipodium formation and stabilization by actin

polymerization [1, 36] (Figure 2A). In addition, it is well established that AQP0 mediates cell-cell adhesion, important for cell integrity [1]. AQPs may also promote cell-matrix adhesion important for tumor cell spread and migration [1], although the involved mechanism remains unclear (Figure 2B). AQPs have also been associated with tumor proliferation by facilitating glycerol uptake, which is essential for cellular biosynthesis and, consequently for cell division [1, 36]. Therefore, AQP expression can be advantageous for high metabolic turnover or tumor-specific metabolic pathways needed for survival of malignant cells. Additionally, the possible interaction between AQPs and oncogenes/ oncoproteins can ultimately activate the transcription of genes involved in cell growth, transformation and survival [1, 36]. The involvement of AQPs in cancer may be also due to the effects triggered by their permeants (Figure 2C). In fact, it was recently reported that some AQP isoforms mediate the uptake of hydrogen peroxide [37, 38], a reactive oxygen species that is involved in intracellular signaling and may promote many aspects of tumor progression [39].

In the last decade, an increasing number of reports showed that AQP5 is abundantly expressed in different tumors and could serve as biomarker with prognostic value of cancer aggressiveness [40-43]. In the next section, we discuss the present knowledge on AQP5 function and regulation as a membrane channel, focusing on AQP5 expression in human cancers, as well as on its implication in cancer biology and involvement in tumor progression.

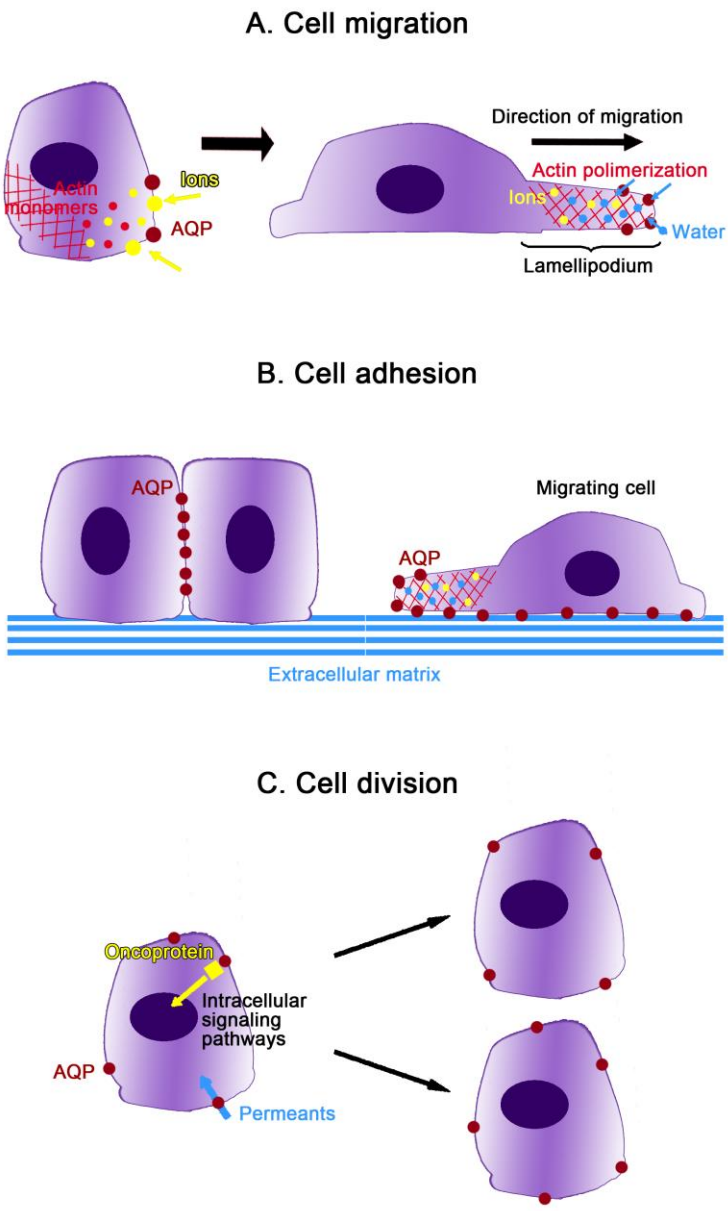


Figure 2 - Aquaporins (AQPs) roles in cancer. (A) AQPs (red dots) polarize at the leading edge of the migrating cell and may facilitate the rapid changes in cell shape by quickly altering transmembrane water fluxes driven by changes in transmembrane ion fluxes, promoting lamellipodium formation and stabilization by actin polymerization. (B) AQP0 plays an important role in cell-cell adhesion. AQPs may also increase cell-matrix adhesion, which is important for tumor spread. (C) AQPs facilitate permeants uptake or may interact with oncoproteins which activate intracellular signalling cascades that promote the transcription of genes involved in tumor cell proliferation.
Adapted from Papadopoulous 2014 [1].

1.2 Aquaporin 5

AQP5 was first cloned from rat submandibular gland [44] and is now known to be widely distributed among the human body, as depicted in Figure 3. In fact, its expression has been described in the digestive [45-47], renal [48], respiratory [49-51], integumentary [52-55], reproductive [56, 57] and nervous [58-62] systems, as summarized in Table 1. Being primarily selective for water, AQP5 thus plays important water flux control throughout the several body systems.

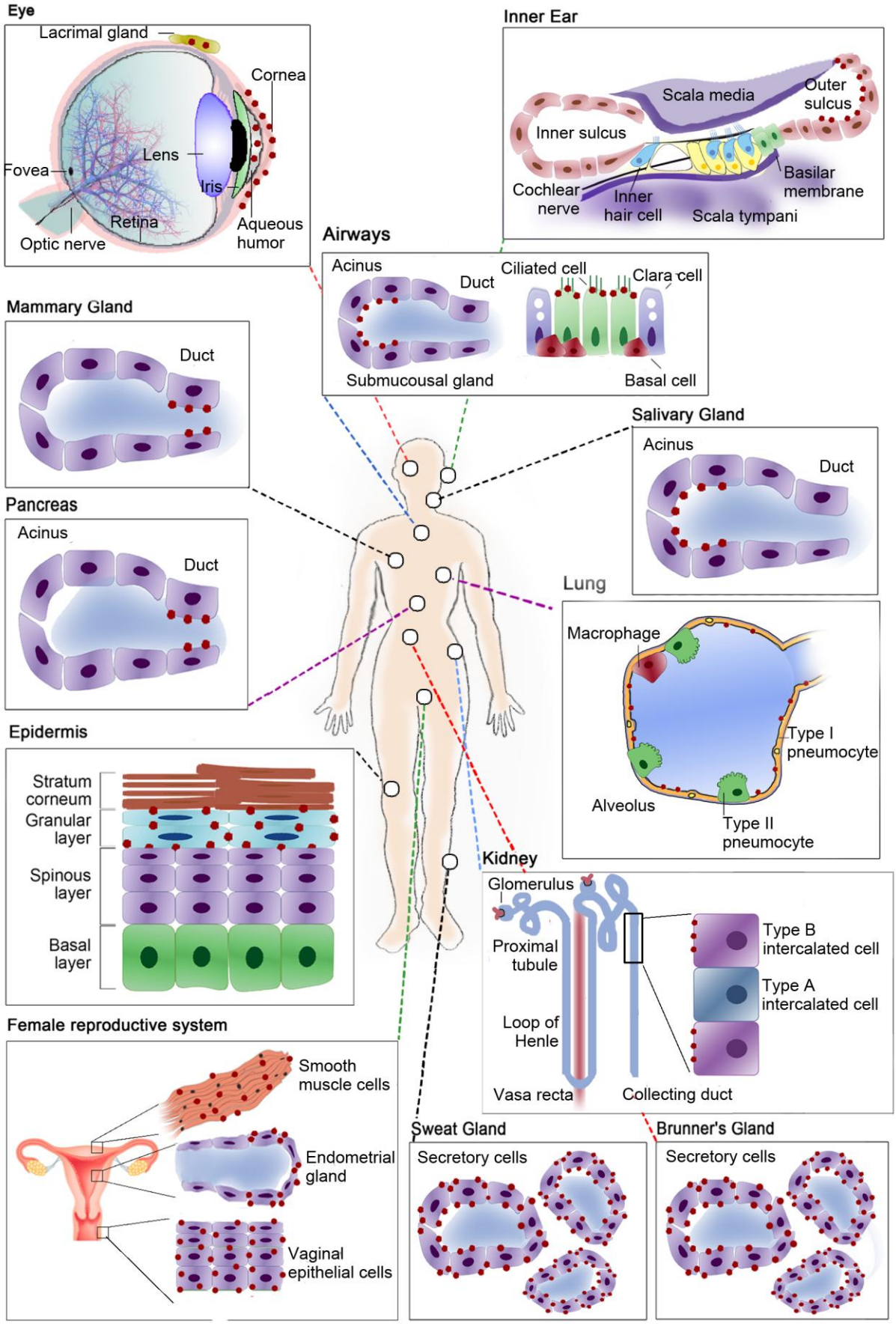

Eye
Lacrimal gland
Cornea
Lens
Fovea
Iris
Retina
Optic nerve
Aqueous humor
Inner Ear
Scala media
Outer sulcus
Inner sulcus
Basilar membrane
Cochlear nerve
Inner hair cell
Scala tympani
Airways
Acinus
Duct
Ciliated cell
Clara cell
Submucousal gland
Basal cell
Mammary Gland
Duct
Salivary Gland
Acinus
Duct
Pancreas
Acinus
Duct
Lung
Macrophage
Type I pneumocyte
Alveolus
Type II pneumocyte
Epidermis
Stratum corneum
Granular layer
Spinous layer
Basal layer
Kidney
Glomerulus
Proximal tubule
Loop of Henle
Vasa recta
Collecting duct
Type B intercalated cell
Type A intercalated cell
Female reproductive system
Smooth muscle cells
Endometrial gland
Vaginal epithelial cells
Sweat Gland
Secretory cells
Brunner's Gland
Secretory cells

Figure 3 - Tissue distribution of human aquaporin 5 (AQP5). In the eye AQP5 is expressed in the cytoplasm and/or plasma membrane of lens fibber cells depending on its differentiation, in the cytoplasm and plasma membrane of corneal epithelium and in the apical membrane of acinar cells in lacrimal glands. In the inner ear this isoform is expressed in the cochlea, in the apical membrane of outer sulcus cells of the apical turn. In the respiratory system AQP5 is expressed in airways at the apical membrane of columnar epithelial cells and at the apical membrane of serous acinar cells of sub-mucosal glands. In lungs, AQP5 is expressed at the apical membrane of type I pneumocytes. It is also expressed in the apical membrane of ductal cells in mammary glands. In the digestive system this isoform is expressed in the pancreas, at the apical membrane of intercalated and intralobular ductal cells and at the apical membrane of acinar cells in salivary glands, as well as at the apical and basolateral membranes of secretory cells of Brunner's glands in the duodenum. In integumentary system AQP5 is expressed in the plasma membrane of keratinocytes in the skin granular layer and in apical and basolateral membranes of secretory cells in sweat glands. AQP5 is also expressed in renal cortex, in the apical membrane of type B intercalated cells in the collecting duct system. In the female reproductive system AQP5 is expressed in the cytoplasm of vaginal epithelial cells, in the basolateral membrane of endometrial glandular epithelial cells and in the plasma membrane of uterus smooth muscle cells. Adapted from Verkman 2014 [17].

Table 1: Tissue distribution and functions of Aquaporin 5 in human body

System	Localization	Subcellular localization	Role	References
Digestive	Pancreas	AP of intercalated and intralobular duct cells; AP of mucoid gland /duct cells	Transcellular water flux for final isotonic pancreatic fluid production	[63]
	Salivary glands: lingual, labial, submandibular and parotid	AP of acinar cells; secretory canaliculi of submandibular and parotid glands	Transcellular water flux for primary saliva fluid production	[64, 65]
	Duodenum: Brunner's glands	AP and BL of secretory cells	Water transport by the gland	[66]
Reprodutive	Vagina	IN in epithelial cells	Transcellular water flux for vaginal lubrification	[56]
	Uterus	PM of smooth	Possible role in	[57, 67]

		muscle cells; BL of glandular epithelial cells; glandular and luminal endometrial cells; stromal cells	transepithelial water flux for uterine secretion and implantation	
Renal	Kidney (cortex)	AP of type B intercalated cells	Transepithelial water reabsorption in collecting system	[48]
Respiratory	Lung	AP of type I pneumocytes	Possible mediation of osmotic water permeability in type I alveolar cells	[49, 50]
	Airway sub-mucousal glands	AP of serous acinar cells	Water flow for surface liquid production and gland homeostasis	[49]
	Trachea	AP of columnar epithelial cells	Replenishment of evaporative losses and rapid restoration to a rehydrated state	[49]
	Bronchi	AP of ciliated duct columnar epithelial cells	Protection of the epithelia from evaporative dehydration and restoration of water from the submucosal vasculature	[49]
	Nasal cavity	AP of columnar epithelial cells facing the lumenal surface	Replenishment of evaporative losses and rapid restoration to a rehydrated state	[49, 51]
Integumentary	Sweat glands	AP and BL of secretory cells	Primary sweat fluid production	[52, 53] [68]
	Skin (stratum granulosum)	PM of keratinocytes	Skin hydration	[55]
	Mammary	AP of epithelial ductal cells	Transcellular water transport in milk production	[69, 70]
	Inner ear (cochlea)	AP of outer sulcus cells of the apical	transcellular water shunt mediation between the	[60]

		turn	perilymph and endolymph	
Senses	Eye (Lens)	IN to PM of fibber cells depending on differentiation	Transparency maintenance of the corneal epithelium as well as underlying stroma; lens homeostasis.	[62]
	Cornea	IN and PM corneal epithelial cell	Corneal fluid elimination	[58, 59, 61]
	Lacrimal glands	AP of acinar cells	Rapid water movement across lacrimal cell membranes	[68]

AP – apical membrane; BL – basolateral membrane; IN - Intracellular; PM – plasma membrane

The 3D structure of AQP5 (Figure 4) shows that this isoform shares the core structural features found in other AQP family members [71]. These channels are inserted in cell membranes as tetramers (Figure 4 A and B) composed by four identical monomers, each behaving as a water pore (Figure 4C). Each monomer interacts with two of its neighbours, forming a central pore (Figure 4 A) that is not involved in water conductance [18], but was suggested to permeate gases [50, 72, 73] and ions [74, 75]. Interestingly, AQP5 structure was solved with a lipid molecule (phosphatidylserine) occluding this central pore [71], whose physiological relevance is still unclearly but may inhibit the permeation of oxygen [76].

The different structural domains of one AQP5 monomer are represented in Figure 4D. Each monomer is composed by 265 amino acids distributed along six transmembrane helices (1-6) connected by five loops, with both amino and carboxyl termini located in the cytoplasm [6, 77]. Loops B and E contain two short hydrophobic stretches of amino acid residues asparagine-proline-alanine (NPA), highly conserved and considered the signature sequence for AQPs [78]. The NPA repeats are embedded in the membrane and are critical to water and substrate permeation. Similarly to other water selective AQPs, AQP5 pore (grey mesh in Figure 4E) contains selective filters with size constrictions and/or charge characteristics that enable water to pass and avoid permeation of solutes above 2.8 Å [79]. Two main regions act as selectivity filters: one is positioned on the extracellular face of the channel in the aromatic/arginine (ar/R) constriction region, and the other is located in the central part of the channel at the NPA region [72, 80, 81] (Figure 4E). These filters also provide a high electrostatic barrier that blocks proton conduction through the pore, which ultimately prevents the dissipation of the electrochemical-potential gradient across the plasma membrane.

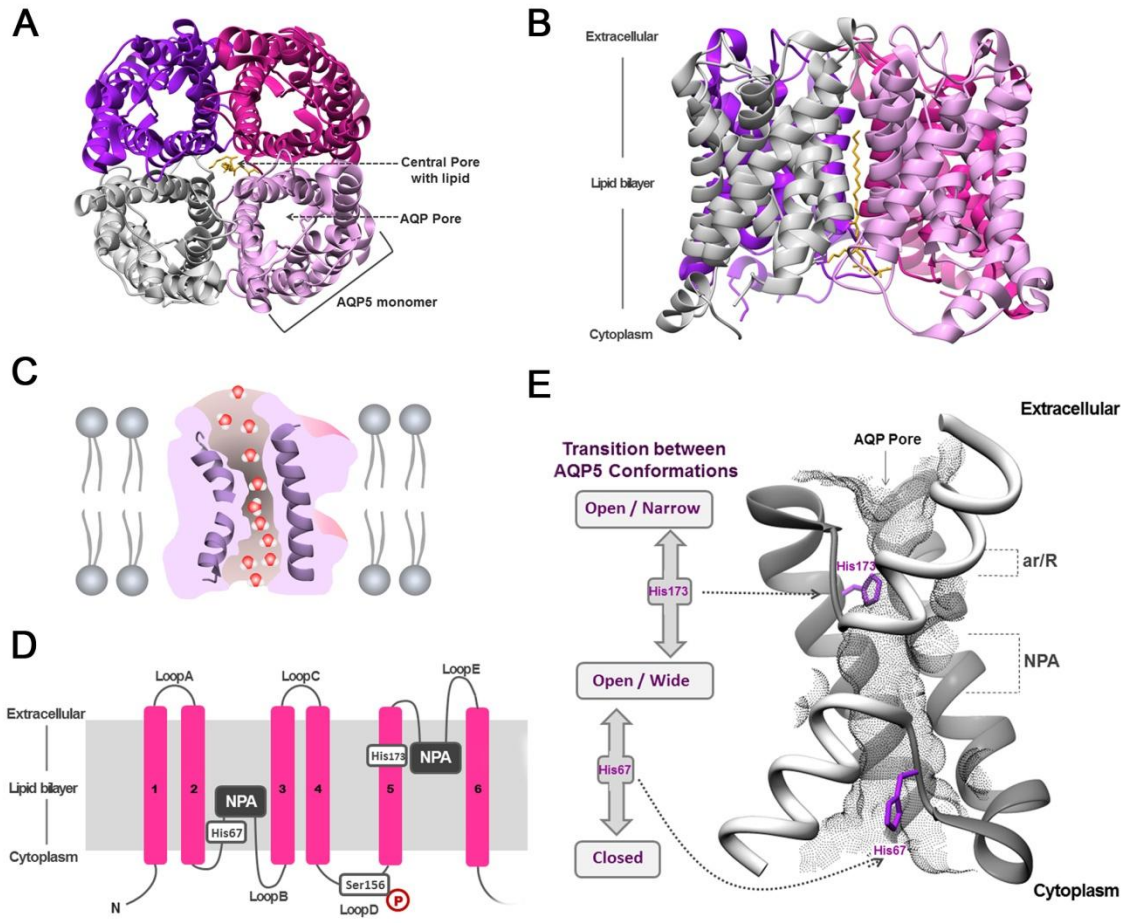


Figure 4 - Structure of AQP5. (A) Extracellular and (B) side views of AQP5 homotetramer, showing phosphatidylserine in the central pore. (C) diagram illustrating how water molecules permeate through AQP pore. (D) topology map of the basic monomeric AQP5 fold, showing the 6 transmembrane α-helices (1-6) connected by loops (A-E), the conserved asparagine-proline-alanine (NPA) motifs embed in the membrane, the histidine residues involved in gating (His 67 and His 173) and the serine residue involved in intracellular signaling (Ser 156). (E) detailed view of AQP5 pore and schematic representation of AQP5 gating mechanism. The two half-helices that form a pseudo seventh transmembrane helix are depicted in white and the positioning of ar/R and NPA selectivity filters is indicated. The grey mesh represents the residues lining AQP5 pore. Key histidine residues involved in AQP5 gating are highlighted: His67, which controls the transition between closed and open conformations, and His173, controlling the transition between wide and narrow states. Structures were generated with Chimera (http://www.cgl.ucsf.edu/chimera) and are based on AQP5 X-ray structure (Protein data bank code: 3D9S).

Similarly to other eukaryotic AQPs, post-transcriptional regulation of AQP5 function has been reported. AQPs are frequently regulated by trafficking, whereby shuttling from intracellular

sites to the plasma membrane occurs in response to various stimuli. The translocation of AQP5 in response to neurotransmitters, hormones [82] and cAMP [83, 84] has been described. Both AQP5 C-terminus [85] and NPA motifs [86] seem to be required for membrane targeting, although the underlying mechanisms still need further elucidation.

AQPs are also regulated by “gating”, which are mechanisms that produce a change in the 3D structure, ultimately affecting the rate of water/solute permeation across the channel [87]. The gating mechanisms regulating human AQP5 activity have been recently disclosed by computational studies [88]. AQP5 channel can switch between different conformations characterized by distinct rates of water flux, thus changing between open and closed, and between wide and narrow conformations, respectively (Figure 4E). The transition between open and closed states occurs through a tap-like mechanism and involves the displacement of the His67 residue located at the cytoplasmic part of the channel. In addition, being in the open conformation, the channel may transition between wide and narrow states, thus conducting maximum and lower water fluxes, respectively. This latter mechanism is governed by the orientation of His173 residue, which is located near the selectivity ar/R filter. The trigger for AQP5 gating is still unclear. It is well established that eukaryotic AQPs can be gated by phosphorylation [87]. Several consensus phosphorylation sites are present in AQP5 [71], however, none of these residues clearly qualifies as the regulatory site [88].

Despite the wide distribution of AQP5 in human body and the detailed information available of its structure and regulatory mechanisms, not much is known about the correlation between AQP5 dysfunction and disease. Recent studies have demonstrated that AQP5 mutations were associated with the development of palmoplantar keratoderma, a disease that is characterized by an abnormal thickening of the skin on the palms of the hands and soles of feet [54, 55]. An AQP5 transport defect was also associated with Sjögren's syndrome, a chronic autoimmune disease that destroys the salivary and lacrimal glands [46, 68, 89]. AQP5 decreased expression in secretory cells of lacrimal and salivary glands probably contribute to the reduced lacrimation and salivation in humans. In addition, *Aqp5-null* mice have reduced saliva and airway submucosal secretions [90] [91], reduced water permeability across the alveolar epithelium [92] and thicker corneas [93]. Therefore, further functional and mechanistic studies are required to disclose AQP5 implication in these pathologies.

1.3 Aquaporin 5 in cancer

Due to AQP5 particular structural characteristics and up-regulation in different tumors, an increasing interest in its involvement in cancer has emerged from 2003 onwards. Table 2 summarizes AQP5 expression profile in human cancers. In general tumor cells overexpress this protein that is primarily located in plasma membranes although also found intracellularly for several cancer types. Recent publications suggest that this isoform enhances cancer cell

proliferation, migration and survival through multiple pathways [94-107] that are not yet fully understood.

Table 2: Aquaporin 5 expression in human cancers

Cancer	Expression	Cellular localization	References
Colon & Colorectal cancer	↑	IN and PM of cancer cells	[96, 99, 108, 109]
Lung ADC	↑	IN and PM of cancer cells	[95, 98, 105, 110-113]
Breast ductal ADC	↑	Invasive cancer cells	[69]
Epithelial ovarian tumors	↑	PM of cancer cells	[102, 103, 114, 115]
Cervical cancer	↑	PM of cancer cells	[116]
Endometrial ADC	↑	Cancer cells	[107]
Esophageal SCC	↑	IN and PM of cancer cells	[117, 118]
Tongue SCC	↑	Cancer cells	[97]
Salivary glands adenoid cystic carcinoma	↓	Cancer cells	[97]
Chronic myelogenous Leukemia	↑	IN and PM of cancer cells - megakaryocytes, myeloblast and granulocytes	[101]
Meningioma	↑	IN of cancer cells	[119]
Astrocytoma	↑	Cancer cells	[120]
Glioblastoma	↑	Fibrous tract possibly along cytoskeleton of cancer cells	[119, 120]
Gastric cancer	↑	PM of cancer cells	[106, 121, 122]
Hepatocellular carcinoma	↑	Cytoplasmic granules and PM of cancer cells	[40]
Gallbladder carcinoma and bile duct carcinoma	↑	PM of cancer cells	[123]
Prostate cancer	↑	Cancer cells	[100]
Pancreatic ductal ADC	ND	Cancer cells	[63]

ADC – Adenocarcinoma; SCC – Squamous cell carcinoma; IN- Intracellular; PM – Plasma membrane; ND - not defined

Figure 5 depicts the proposed molecular events accounting for AQP5 involvement in tumorigenesis. In tumors, the cyclic adenosine monophosphate (cAMP) dependent phosphorylation of AQP5 on Ser156 by Protein kinase A (PKA) activates the RAS/Mitogen-activated protein kinases (MAPK) pathway involved in cell proliferation and survival [94, 95]. AQP5 also binds to the SH3 domain of adaptor molecules, such as c-Src, Lyn and Grap-2-C-terminal [95] that can in turn activate intracellular pathways, such as RAS/MAPK [94-96]. AQP5 seems only to interact with the activated form of c-Src [95], which is associated with epithelial mesenchymal transition (EMT), a common process in invasive tumors by which cells lose their epithelial characteristics and acquire migratory mesenchymal properties [35], starting to express mesenchymal markers [35] and presenting a marked spindle cell-like/ fibroblastic phenotype [95]. Activated c-Src promotes EMT by disrupting E-cadherin dependent cell-cell contact and enhancing integrin mediated cell-matrix adhesion [124]. The activation of integrins and focal adhesion kinase (FAK)-MAPK signalling may also play a prominent role in tumor spread related with AQP5 up-regulation [125] AQP5 may additionally facilitate cancer cell motility due to its preferential polarization in the leading edge of migrating cells [100, 102, 105-107], as described for other oncogenic AQPs [1] (represented in Figure 2A).

Microarray analysis after AQP5 silencing revealed its influence on the expression of genes related with cell growth, development, cycle progression and apoptosis [118]. This agrees with higher susceptibility to apoptosis after down-regulating AQP5 in cancer cells [101]. The link between AQP5 and tumor resistance to apoptosis is unclear, but may involve the activation of the epidermal growth factor receptor (EGFR). Up-regulating AQP5 in lung cancer cells activated EGFR [105], which is known to trigger the RAS/MAPK as well as phosphatidylinositol-3-kinase (PI3K)/AKT signal pathways [126]. PI3K activates AKT that in turn blocks caspase-9, ultimately blocking apoptosis in AQP5 expressing cancer cells [101]. It is also known that AQP5 promoter contains sequences for nuclear factor-κB (NF-κB) [127], suggesting that NF-κB may regulate AQP5 expression [103, 104]. In the cytoplasm NF-κB binds to its inhibitor IκBα and stays inactive. After receptor activation, IκBα is degraded and NF-κB translocates into the nucleus where it activates the transcription of AQP5 and anti-apoptotic genes, promoting cell proliferation and survival [103, 104].

In colon cancer cells, AQP5 also associated with the p38 MAPK pathway [99], which is activated in response to stress signals, like chemotherapy-damaged DNA. Activation of p38 MAPK pathway leads to the expression of multidrug resistance proteins responsible for tumor drug resistance [99, 108, 128].

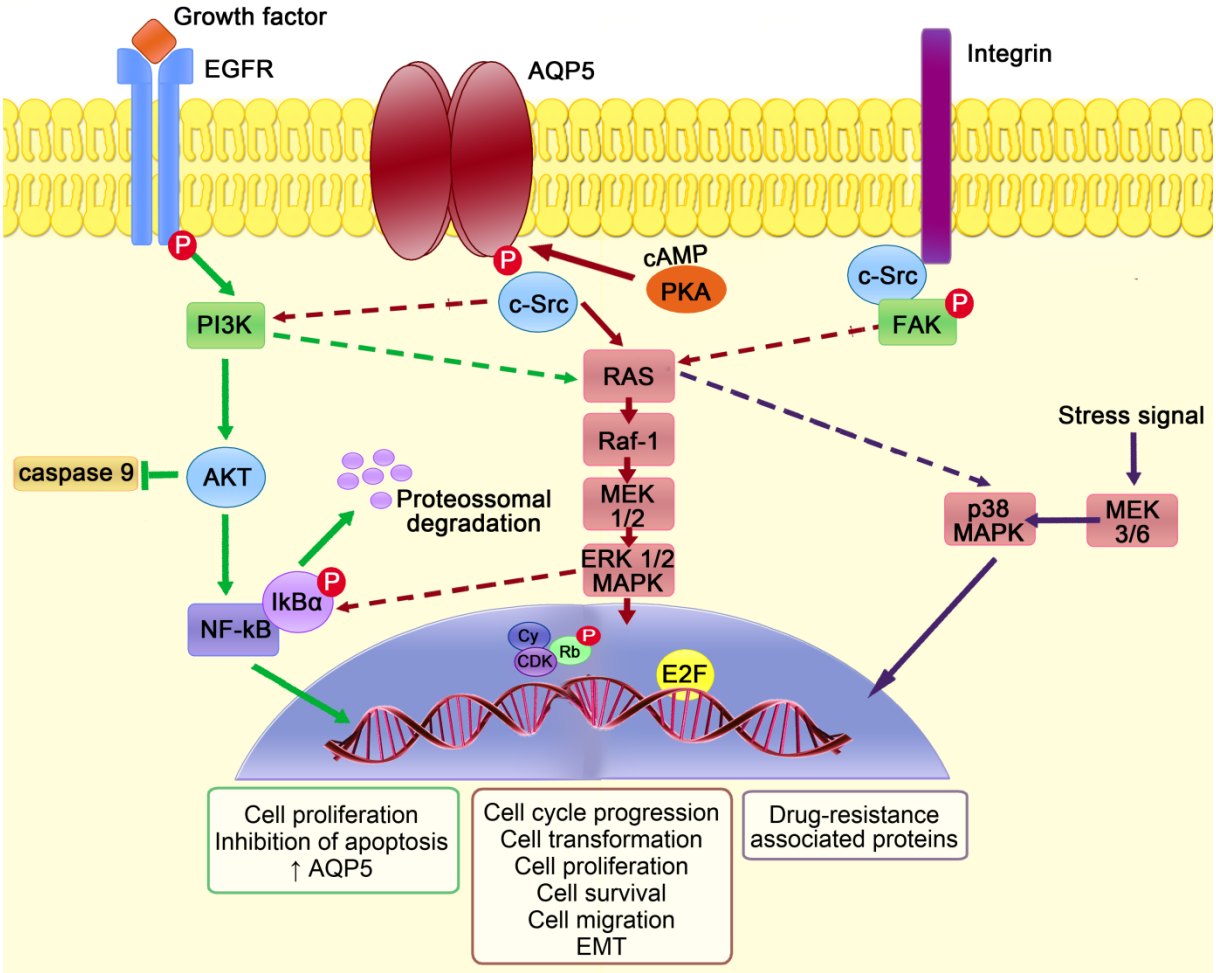


Figure 5 - AQP5 intracellular signalling pathways involved in cancer. Phosphorylation of AQP5, mediated by cAMP-dependent protein kinase A (PKA), promotes the binding of adaptor molecules with SH3 domain, such as c-Src, triggering intracellular signalling cascades. Downstream, there is signal transduction through RAS / Raf-1 / MEK1/2 / ERK1/2 pathway to the nucleus, where cyclin (Cy)/ Cyclin-dependent kinases (CDK) complexes phosphorylate retinoblastoma protein (Rb) that releases the transcription factor E2F, leading to expression of genes that are involved in cell transformation, proliferation, cycle progression and survival. Cell-matrix adhesion mediated by integrins is required for the continuous activation of FAK-MAPK pathway that is essential for cytoskeleton integrity. This pathway also promotes epithelial mesenchymal transition (EMT) by increasing the expression of mesenchymal markers and decreasing the expression of epithelial markers, essential for cancer cell migration and spread. Epidermal growth factor receptor (EGFR) activation by growth factors can activate RAS /MAPK signal transduction pathway and PI3K, which in turn activate AKT that is able to block caspase-9 activation. In the cytoplasm, nuclear factor-κB (NF-κB) binds to its inhibitor IκBα and stays in an inactive state. After receptor activation, IκBα is phosphorylated and undergoes proteossomal degradation. NF-κB is released and translocates into the nucleus where it activates the transcription of target

genes, namely anti-apoptotic genes, resulting in increased AQP5 expression, cell proliferation and survival. AQP5 expression was also recently implicated in colon cancer multidrug resistance mechanisms due to activation of p38 MAPK pathway in response to stress signals.

1.4 Pancreatic Cancer

Pancreatic cancer (PC) is the seventh leading cause of cancer mortality in the world with an estimated 5-year prevalence of 4,1 per 100,00 people [129, 130]. More than 85% of the pancreatic tumors are invasive ductal adenocarcinomas (PDA) and are located in the head of the pancreas (>60%) [131, 132]. Depending on the degree of differentiation (well to poorly) PDA may show, embedded in a desmoplastic stroma, well to poorly formed glands or individual infiltrating cells forming sheets [131, 132]**.** Poorly differentiated PDA are more aggressive and, consequently fatal tumors than the moderately differentiated ones [131]. Due to the retroperitoneal location of the pancreas, the early stages of this type of cancer do not usually produce symptoms. The disease is generally advanced when it is diagnosed and more than 50% of patients have distant metastasis so, 5-year survival rate from all stages of PC is approximately 6% [130]. Therefore, the need for useful biomarkers has been consistently suggested to detect PC in early stages.

Although AQP5 normal expression at the apical membrane of intercalated and intralobular pancreatic ductal cells [63, 89, 133], little is known about its expression and roles in pancreatic ductal adenocarcinoma (PDA). Therefore, the aim of this study was to investigate the expression pattern of AQP5 in PDA and its clinical significance. To evaluate the potential of AQP5 as a novel biomarker for PDA, we performed immunohistochemical studies in human PDA and control samples and evaluated the relationship between AQP5 expression and a proliferative cell marker (Ki-67), a PDA marker (Cytokeratin 7), an epithelial cell marker (E-cadherin) and a mesenchymal cell marker (Vimentin).

2. Materials and Methods

2.1 Patients

Formalin-fixed and paraffin embedded tumor samples and matched adjacent non-neoplastic samples were obtained from 35 patients who underwent curative surgery for PDA at Curry Cabral Hospital, Centro Hospitalar de Lisboa Central, Lisbon, Portugal, between November 2012 and March 2015. The patients' clinicopathological features are summarized in Table 3. All patients were classified according to the pathological tumor/node/metastasis (pTNM)

system and based on the 7th edition of America Joint Committee on Cancer (AJCC) the pathologic stages after surgical resection were stages I and II. Clinicopathological features were reviewed for sex, histological differentiation, original tumor dimension, tumor location, lymph node metastasis, perineural, vascular and intestinal invasion. Samples were fixed with 4% formalin and processed for histological studies.

Table 3: Clinicopathological features of the 35 patients analysed

Case	Sex	Histological Differentiation	Stage	TNM	Dimension (cm)	Lymph node metastasis	Perineural invasion	Vascular invasion	Intestinal invasion
1	F	Moderatly	II	pT3 N0 Mx	3x3,2x2,4	No	No	No	Yes
2	M	Moderatly	I	pT2 N0 Mx	1,5x1,5x2	No	No	No	Yes
3	M	Moderatly	II	pT3 N1	2,5x1x0,3	Yes	Yes	No	No
4	F	Moderatly	II	pT3 N1	2,5x2,5x2	Yes	Yes	No	No
5	M	Poorly	II	pT3 N1 Mx	0,5x1x 0,4	Yes	Yes	Yes	Yes
6	F	Moderatly	II	pT3 N0 Mx	3x2,5x2,5	No	Yes	No	Yes
7	F	Moderatly	II	pT2 N1	1,5x1	Yes	No	No	Yes
8	M	Poorly	II	pT3 N1	3,5x2,5x2	Yes	Yes	Yes	Yes
9	M	Moderatly	I	pT2 N0 Mx	4,5x3x2	No	No	No	Yes
10	M	Poorly	II	pT3 N0 Mx	4x3x2,5	No	Yes	No	No
11	F	Moderatly	II	pT3R1, N1	4,2x4x2,5	Yes	Yes	No	No
12	M	Moderatly	II	pT3 N1 Mx	1x1,5x1	Yes	No	Yes	No
13	M	Poorly	II	pT3 N1 Mx	3x2x2	Yes	No	No	No
14	F	Moderatly	II	pT3 N0 Mx	2x1,2x1	No	No	No	Yes
15	M	Poorly	II	pT3 N1 Mx	4x3,5x3	Yes	Yes	Yes	Yes
16	F	Poorly	II	pT3 N1 Mx	0,4	Yes	Yes	Yes	Yes
17	M	Moderatly	II	pT3 N1 Mx	3x2,5x2,5	Yes	Yes	Yes	Yes
18	M	Poorly	II	pT3 N1 Mx	1,5	Yes	Yes	Yes	No
19	F	Moderatly	II	pT3 N1 Mx	2,5x1,5x3	Yes	Yes	Yes	Yes
20	F	Moderatly	II	pT3 N1 Mx	2,7x2x2,2	Yes	Yes	Yes	No
21	F	Moderatly	II	pT3 N1 Mx	3,5x3x2,5	Yes	Yes	No	Yes
22	F	Poorly	II	pT3 N1 Mx	4,5x3,5x4	Yes	Yes	Yes	Yes
23	M	Moderatly	II	pT3 N1 Mx	2	Yes	No	No	Yes
24	M	Moderatly	II	pT3 N1 Mx	4x3,5x2,5	Yes	No	No	Yes
25	F	Poorly	II	pT3 N1 Mx	3,5x3,3x3	Yes	Yes	No	No
26	M	Poorly	II	pT3 N1 Mx	2,5x2,5x2,2	Yes	No	No	Yes
27	F	Poorly	II	pT2 N1 Mx	2,5x3x2,5	Yes	No	No	No
28	M	Moderatly	II	pT3 N1 Mx	2,5	Yes	Yes	Yes	No
29	F	Moderatly	II	pT3 N1 Mx	4X2,7X2,5	Yes	Yes	Yes	Yes
30	M	Moderatly	II	pT3 N1 Mx	2x2x0,4	Yes	Yes	No	No
31	M	Moderatly	I	pT2 N0 Mx	2x1	No	No	No	Yes
32	M	Moderatly	II	pT3 N1 Mx	3x2	Yes	No	No	Yes
33	F	Poorly	II	pT3 N0 Mx	2x1,7x1,5	No	Yes	No	No
34	M	Moderatly	I	pT2 N0 Mx	2,5x1,5	No	No	No	Yes
35	F	Moderatly	II	pT3 N0 Mx	2x2,5	No	Yes	No	No

F- Female; M- Male

2.2 Immunohistochemistry

Immunohistochemistry was performed on 4 μm paraffin sections of pancreas as summarized in Table 4. A 0.3% hydrogen peroxide solution was used to inhibit endogenous peroxidase, for 30 min, and antigen retrieval was achieved by heat-mediated treatment with citrate buffer pH6.Sections were incubated overnight at 4ºC with primary antibodies. A post-incubation step was only performed for Ki-67 antibody. Sections were incubated with SuperPicture™ Polymer Detection Kit (Abcam), followed by development with 3,3-diaminobenzidine tetrahydrochloride and counterstaining with haematoxylin. Negative controls with omission of primary anti-bodies were performed to exclude nonspecific binding or cross reactivity.

Table 4: Immunohistochemistry conditions

Markers	Antigen retrieval	Primary antibody	Dilution	Incubation time	Post-incubation	Secondary antibody
AQP5	MW (800W) for 10' in citrate buffer pH6	Abcam, #ab92320 Rabbit Mc	1:250	Overnight at 4ºC	NA	Abcam, SuperPicture™ Polymer Detection Kit
Ki-67	MW (800W) for 20' in citrate buffer pH6	Invitrogen, #18-0192Z Mouse Mc	1:50	48h at 4ºC	1h at 37ºC	Abcam, SuperPicture™ Polymer Detection Kit
CK7	MW (800W) for 10' in citrate buffer pH6	Abcam, # ab181598 Rabbit Mc	1:4000	Overnight at 4ºC	NA	Abcam, SuperPicture™ Polymer Detection Kit
Vim	MW (800W) for 10' in citrate buffer pH6	Abcam, # ab92547 Rabbit Mc	1:500	Overnight at 4ºC	NA	Abcam, SuperPicture™ Polymer Detection Kit
E-cad	MW (800W) for 40' in citrate buffer pH6	Abcam, #ab40772 Rabbit Mc	1:400	Overnight at 4ºC	NA	Abcam, SuperPicture™ Polymer Detection Kit

AQP5, Aquaporin 5; CK7, Cytokeratin 7; Vim, Vimentin; Ecad, E-cadherin; #, catalog reference; Mc, Monoclonal; NA, not applicable

2.3 Scoring of Immunohistochemistry

The immunohistochemical analysis was graded based on the staining intensity (SI) and percentage of positive cells (PPC) by one investigator that was blinded to the clinicopathological variables. The SI was scored on a scale of four grades: 0, no staining; 1, weak staining; 2, moderate staining and 3, strong staining. The PPC was graded on a scale of 3grades: 0, <10% stained cells; 1, ≥10% <50% stained cells and 2, ≥50% stained cells. Expression of AQP5, Ki-67, CK7, E-cad and Vim were defined as final immunohistochemistry score (FIS) based on the sum of the SI and PPC. AQP5, Ki-67, CK7, E-cad and Vim expression in each PDA sample was considered increased or decreased when FIS was higher or lower, respectively, than the mean FIS value for control samples. Each PDA

sample was considered higher or lower AQP5, Ki-67, CK7, E-cad and Vim expresser when FIS were higher or lower, respectively, than the mean FIS value for PDA samples.

2.4 Statistical Analysis

The descriptive statistics are reported as proportions. The baseline characteristics and results of the immunohistochemical staining were compared using a Fisher's exact test. Paired Student's *t*-test was used to compare the expression levels between tumor and normal (control) pancreatic tissues. The correlation between AQP5, Ki-67, CK7, E-cad and Vim expression in pancreatic ductal adenocarcinoma tissues were assessed using the Spearman rank correlation test. Statistical analysis was performed using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA) and P <0.05 was considered significant.

3. Results

3.1 Overexpression of AQP5, Ki-67, CK7, E-cad and Vim in PDA

The expression levels, evaluated as FIS values, of AQP5, Ki-67, CK7 and Vim in PDA tissues were significantly higher than those in adjacent normal pancreatic tissues (p <0.001, Table 5). On the contrary, E-cad protein levels were found to be significantly down-regulated in PDA samples (p <0.001, Table 5). The mean FIS values for AQP5, Ki-67, CK7, E-cad and Vim were 4.06, 1.94, 4.09, 3.00 and 4.77, respectively. Of the 35 PDA cases investigated, 27 (77.14%) were regarded as AQP5 high expression group, 19 (54.29%) as Ki-67 high expression group, 30 (85.71%) as CK7 high expression group, 6 (17.14%) as E-cad low expression group and 27 (77.14%) as Vim high expression group (Table 6).

The Spearman's rank correlation analysis showed that the expression level of Ki-67 was closely correlated with that of Vim (Spearman's r = 0.373; P=0.027, Table 7).

Table 5: Comparison of Aquaporin 5 (AQP5), Ki-67, Cytokeratin 7 (CK7), Vimentin (Vim) and E-cadherin (E-cad) expression levels between pancreatic ductal adenocarcinoma (PDA) and adjacent non-neoplastic (control) samples.

Groups	AQP5		Ki-67		CK7		E-cad		Vim	
	Mean	*P value*	Mean	*P value*	Mean	*P value*	Mean	*P value*	Mean	*P value*
PDA	4.06	**<0.001**	1.94	**<0.001**	4.09	**<0.001**	3.00	**<0.001**	4.77	**<0.001**
Control	1.57		0.09		2.51		4.49		3.20	

Table 6: Counting of high and low Aquaporin 5 (AQP5), Ki-67, Cytokeratin 7 (CK7), E-cadherin (E-cad) and Vimentin (Vim) expresser pancreatic ductal adenocarcinoma (PDA) cases.

	Ki-67		CK7		E-cad		Vim	
	< 2 (n=16) 45.71%	≥ 2 (n=19) 54.29%	< 4 (n=5) 14.29%	≥ 4 (n=30) 85.71%	< 3 (n=6) 17.14%	≥ 3 ´ (n=29) 82.86%	< 5 (n=8) 22.86%	≥ 5 (n=27) 77.14%
AQP5								
< 4 (n=8) 22.86%	5	3	1	7	1	7	3	5
≥ 4 (n=27) 77.14%	11	16	4	23	5	22	5	22

Limits presented in the table are based on mean FIS values for AQP5 (4.06), Ki-67 (1.94), CK7(4.09), E-cad (3.00) and Vim (4.77) in PDA samples.

Table 7: Association between Aquaporin 5 (AQP5), Ki-67, Cytokeratin 7 (CK7), E-cadherin (E-cad) and Vimentin (Vim) expression in pancreatic ductal adenocarcinoma (PDA) samples.

	Spearman's r	AQP5	Ki-67	CK7	E-cad	Vim
AQP5	Correlation Coefficient		0,243	0,080	0,236	0,082
	p value		0,159	0,646	0,173	0,639
Ki-67	Correlation Coefficient	0,243		-0,035	0,021	**0,373**
	p value	0,159		0,842	0,907	**0,027**
CK7	Correlation Coefficient	0,080	-0,035		-0,131	0,243
	p value	0,646	0,842		0,455	0,160
E-cad	Correlation Coefficient	0,236	0,021	-0,131		-0,232
	p value	0,173	0,907	0,455		0,179
Vim	Correlation Coefficient	0,082	**0,373**	0,243	-0,232	
	p value	0,639	**0,027**	0,160	0,179	

3.2 Immunoexpression patterns of AQP5, Ki-67, CK7, E-cad and Vim in PDA and adjacent non-neoplastic samples

In normal pancreatic tissues AQP5 is expressed in the apical membrane of intercalated and intralobular ductal cells (Figure 6 A and B). In PDA AQP5 is expressed all over the plasma membrane and becomes to diffuse intracellularly (Figure 6 C to E). Moderately differentiated PDAs show a stronger AQP5 immunoreactivity when compared with poorly differentiated ones. Surprisingly, AQP5 expression was also detected in the plasma membrane of some scattered cells in duodenal epithelium that do not express this protein in normal conditions (Figure 6 F and G). This isoform is also expressed in apical membrane of Brunner's glands cells (Figure 6G). The frequencies of AQP5 FIS can be consulted in graph 1. For detailed

information about FIS, SI and PPC parameters please see appendix material (graphs 6, 7 and 8).

Ki-67 nuclear expression is almost absent in normal pancreatic tissue except for some rare mitotic figures as depicted in Figure 7A and B. In PDA samples Ki-67 protein levels tend to be very heterogeneous: in some tumors it is possible to observe many proliferative cells while in others just a few cells are stained (Figure 7C to F). The expression of this protein was only detected in the base of duodenal crypts (Figure 7 G and H). The frequencies of Ki-67 FIS can be consulted in graph 2. For detailed information about FIS, SI and PPC parameters please see appendix material (graphs 9, 10 and 11).

CK7 was found to be expressed in the plasma membrane of ductal cells in normal pancreas (Figure 8 A and B). In PDA cells this cytokeratin is expressed intracellularly and in the plasma membrane (Figure 8 C to F). Interestingly in duodenum, although with weaker immunolabelling, CK7 was detected in the plasma membrane and in intracellular vesicles of the same scattered cells that also express AQP5 (Figure 8 G and H). The frequencies of CK7 FIS can be consulted in graph 3. For detailed information about FIS, SI and PPC parameters please see appendix material (graphs 12, 13 and 14).

E-cad is widely expressed in intercellular junctions of non-neoplastic tissues (Figure 9 A and B). The expression of this protein is decreased in PDA with some cells do not showing any staining in intercellular junctions which is more evident in poorly differentiated tumors (Figure 9 C to F). E-cad is also widely expressed in intercellular junctions of epithelial cells of duodenum (Figure 9 and H). The frequencies of E-cad FIS can be consulted in graph 4. For detailed information about FIS, SI and PPC parameters please see appendix material (graphs 15, 16 and 17).

In non-neoplastic samples Vim is expressed in non-epithelial cells, such as fibroblasts and lymphoid cells (Figure 10A and B). This protein is abundantly expressed in tumoral desmoplastic stroma (Figure 10C to F). Vim expression was also found in mesenchymal cells in duodenum, in the plasma membrane of lymphocytes and in some scattered cells in duodenal epithelium (Figure 10G and H). The frequencies of Vim FIS can be consulted in graph 5. For detailed information about FIs, SI and PPC parameters please see appendix material (graphs 18, 19 and 20).

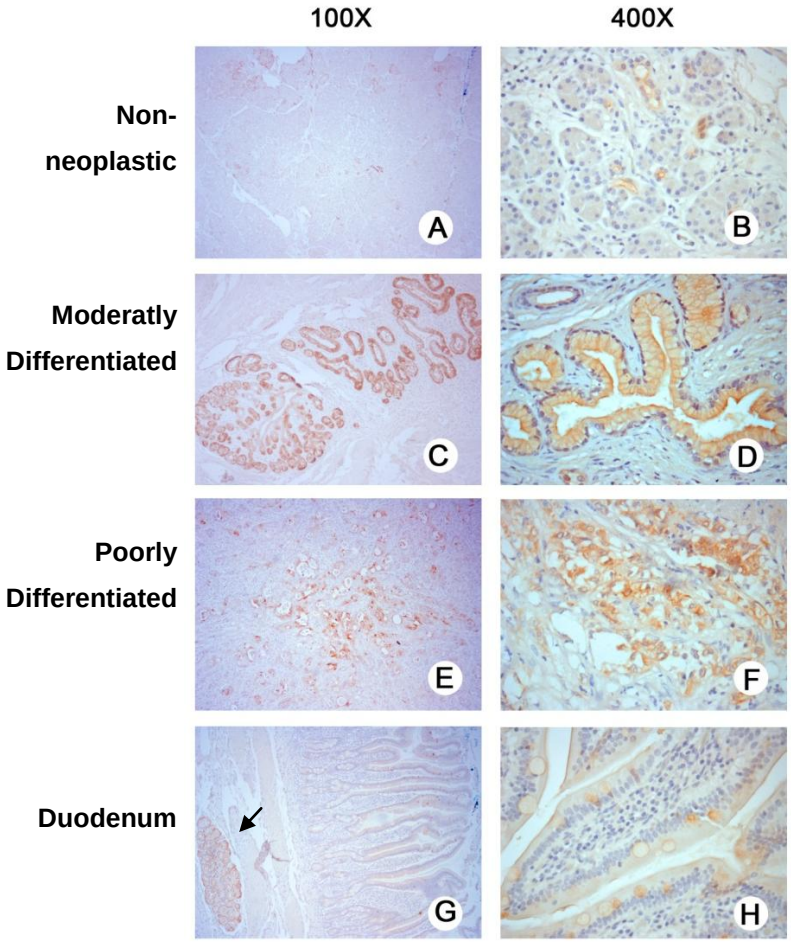
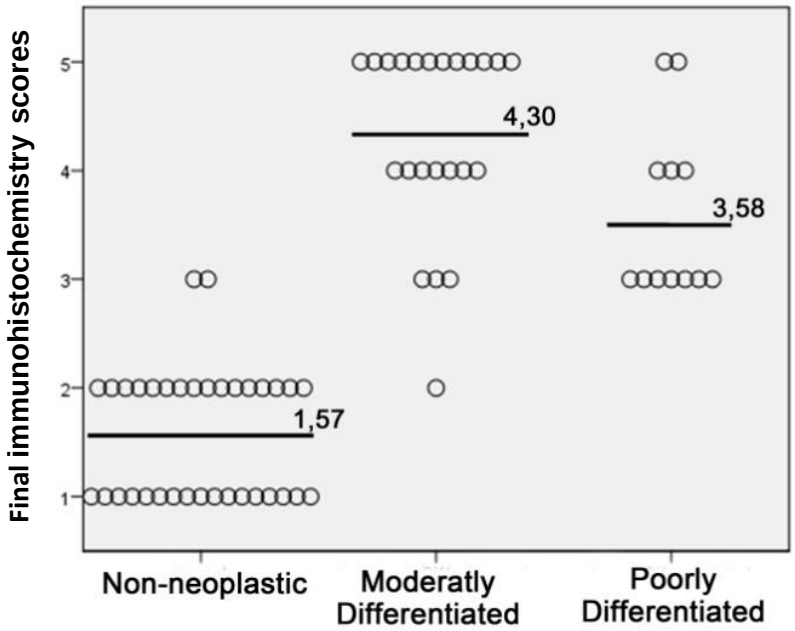


Figure 6 - Aquaporin 5 (AQP5) expression in non-neoplastic, pancreatic ductal adenocarcinoma (PDA) and duodenum samples. AQP5 is expressed in the apical membrane of intercalated and intralobular ductal cells (A) and (B); AQP5 is expressed in the plasma membrane and becomes to diffuse intracellularly in PDA cells (C) to (F); AQP5 expression was also detected in Brunner's glands (black arrow) and in some vacuolated cells scattered in duodenum (G) and (H);



Graph 1 - Frequencies of Aquaporin 5 final immunohistochemistry scores. Mean value in non-neoplastic, moderately and poorly differentiated PDA samples is 1,57, 4,30 and 3,58; respectively.

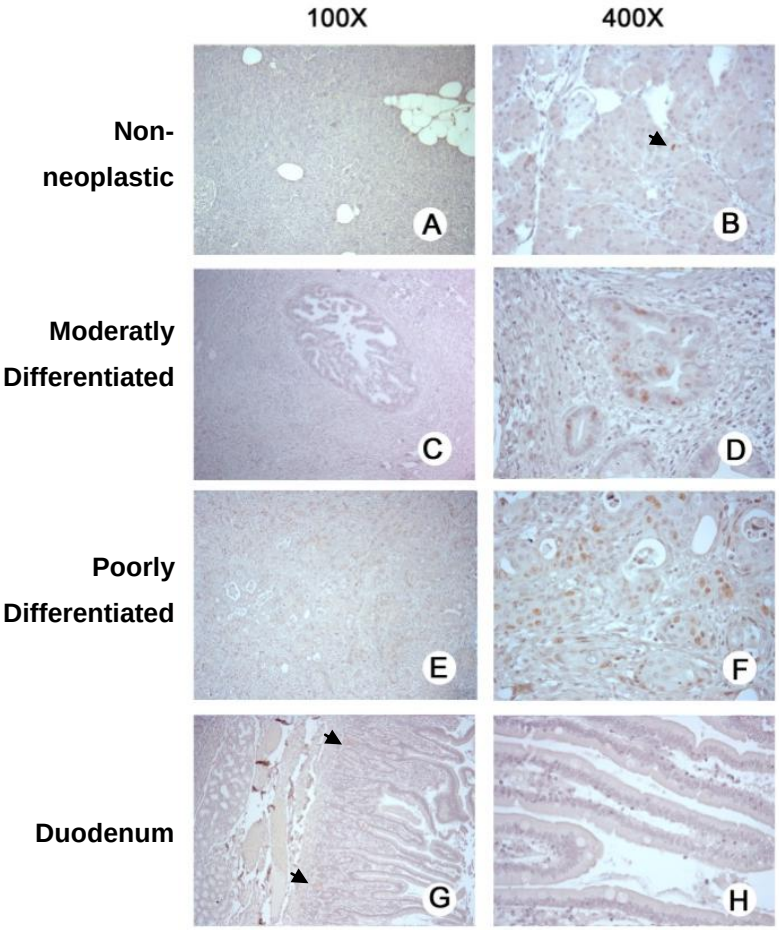
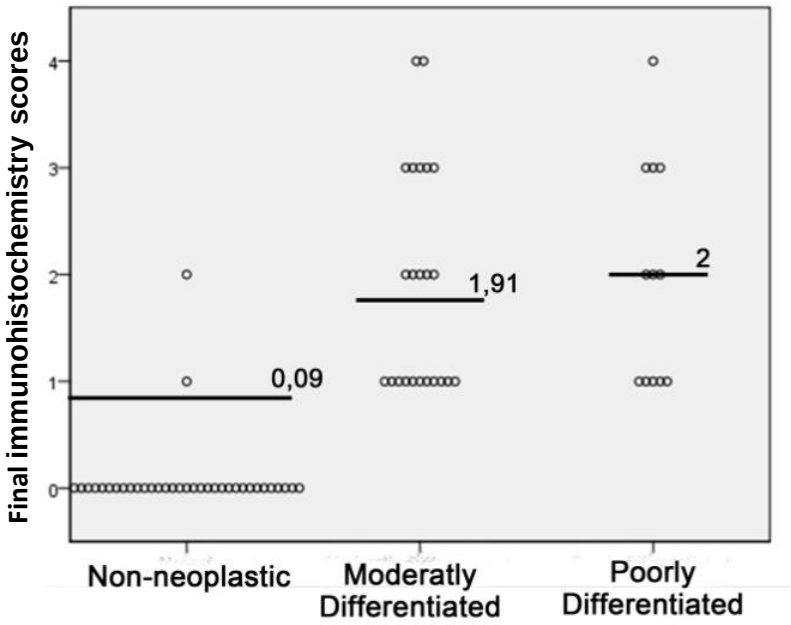


Figure 7 - Ki-67 expression in non-neoplastic, pancreatic ductal adenocarcinoma (PDA) and duodenum samples. Ki-67 expression is almost absent in normal pancreatic tissue (A) except for some rare mitotic figures (B, black arrow); Ki-67 nuclear staining shows proliferative ductal carcinoma cells (C) to (F); The expression of this protein is also detected in the base of duodenal crypts (G, black arrows) but it is absent in duodenal epithelial cells (H).



Graph 2 - Frequencies of Ki-67 final immunohistochemistry scores. Mean value in non-neoplastic, moderately and poorly differentiated PDA samples is 0,09, 1,91 and 2,00; respectively.

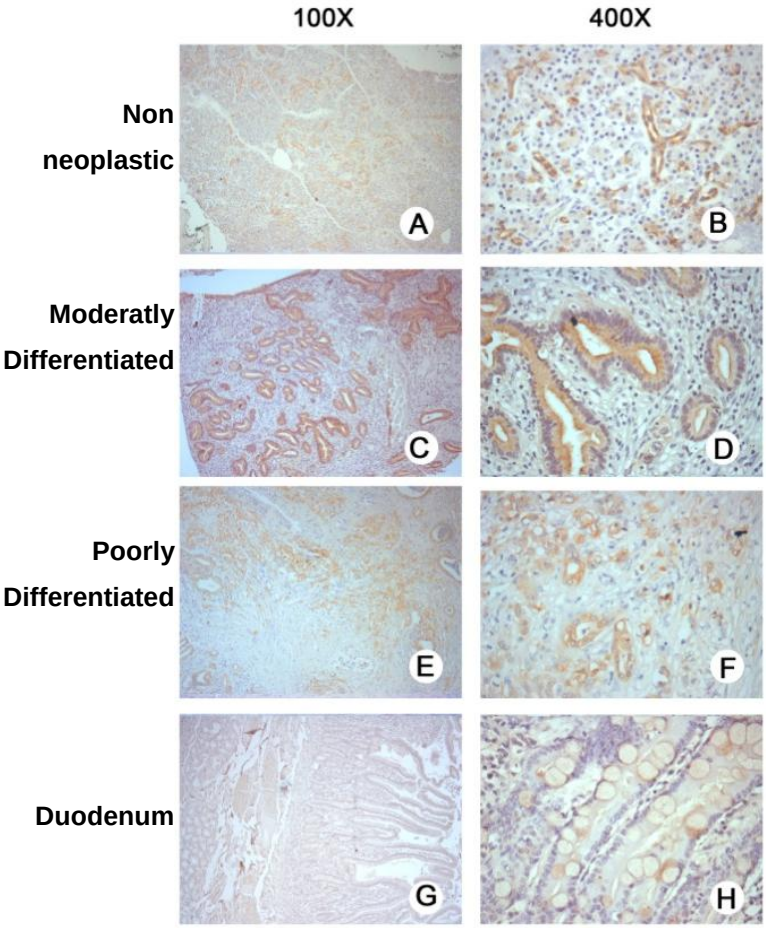
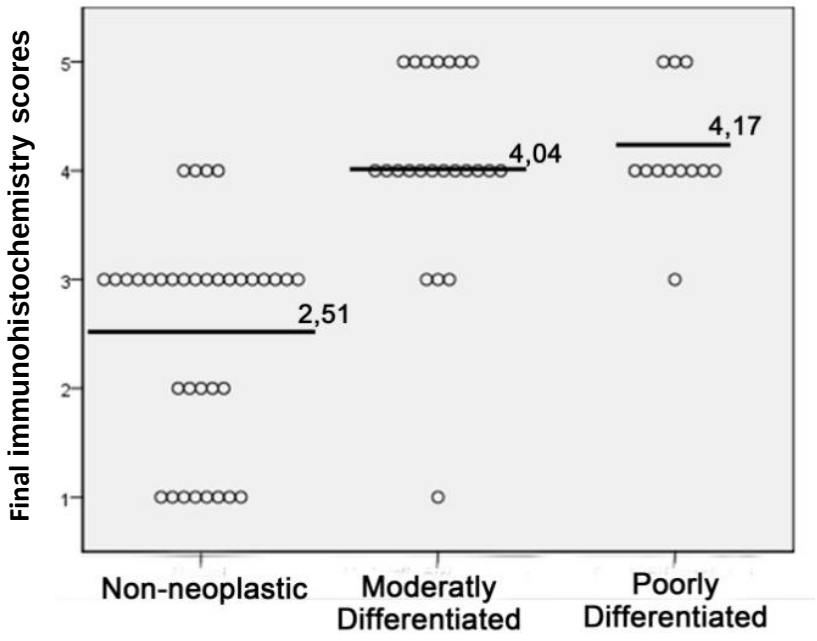


Figure 8 - Cytokeratin 7 (CK7) expression in non-neoplastic, pancreatic ductal adenocarcinoma (PDA) and duodenum samples. CK7 is expressed in the plasma membrane of ductal cells on non-neoplastic pancreas (A) and (B); This cytokeratin is also expressed in the plasma membrane and intracellularly in PDA cells (C) to (F); CK7 was detected in the plasma membrane and in intracellular vesicles of the same scattered cells that also express AQP5, although with weaker immunostaining (G) and (H).



Graph 3 - Frequencies of Cytokeratin 7 final immunohistochemistry scores. Mean value in non-neoplastic, moderately and poorly differentiated PDA samples is 2,51, 4,04 and 4,17, respectively.

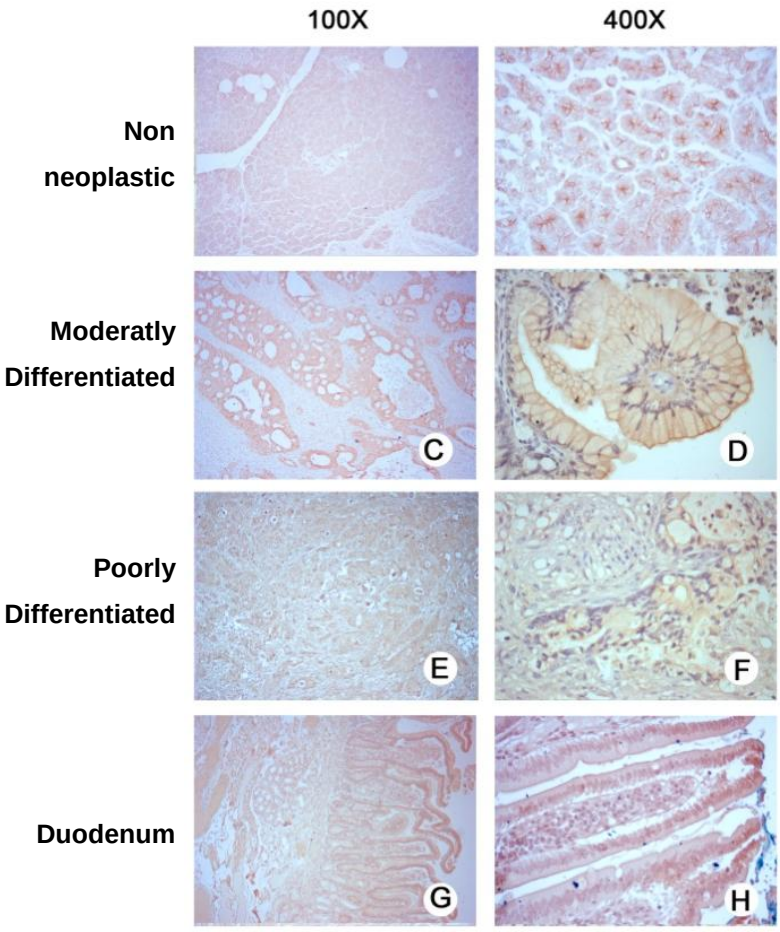
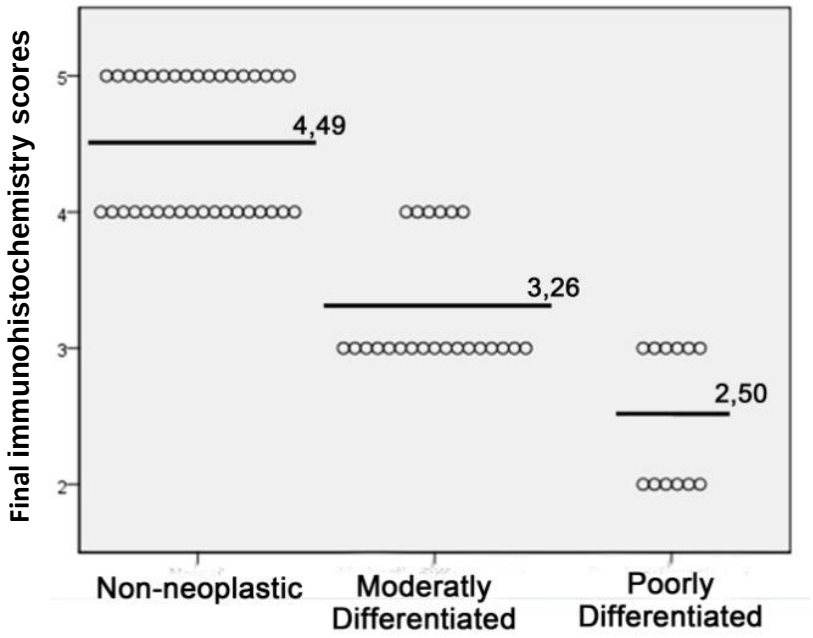


Figure 9 - E-cadherin (E-cad) expression in non-neoplastic, pancreatic ductal adenocarcinoma (PDA) and duodenum samples. E-cad is widely expressed in non-neoplastic tissue in intercellular junctions (A) and (B); E-cad expression is decreased in intercellular junctions of moderately differentiated adenocarcinoma duct cells (C) and (D) and even more decreased in poorly differentiated tumors (E) and (F); This protein is also widely expressed in intercellular junctions of epithelial duodenal cells (G) and (H).



Graph 4 - Frequencies of E-Cadherin final immunohistochemistry scores. Mean value in non-neoplastic, moderately and poorly differentiated PDA samples is 4,49, 3,26 and 2,5, respectively

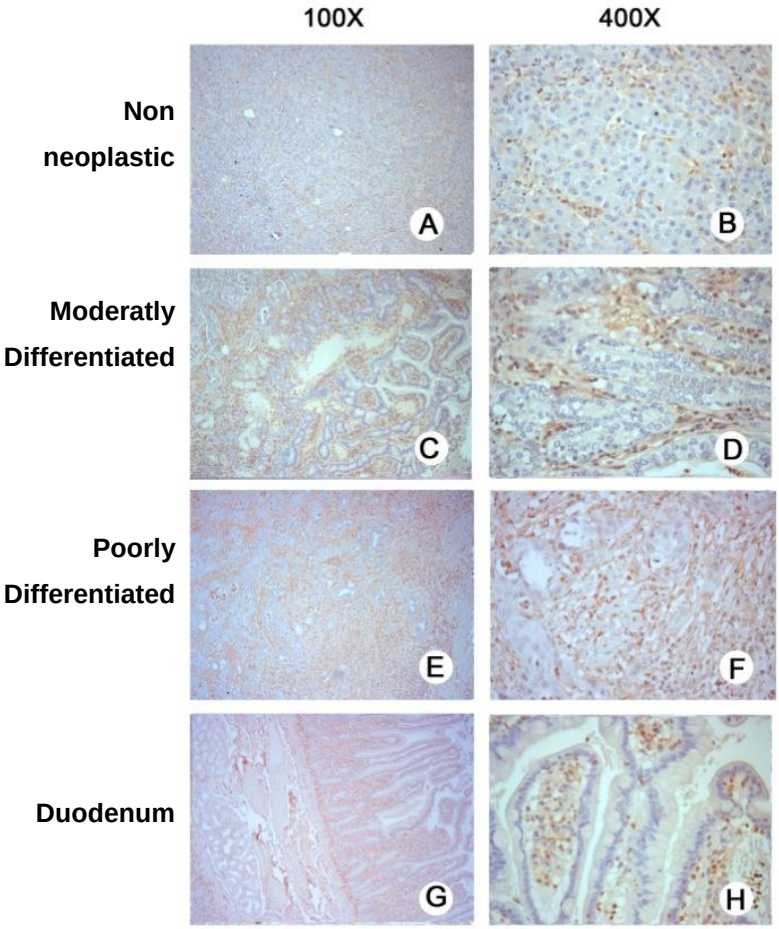
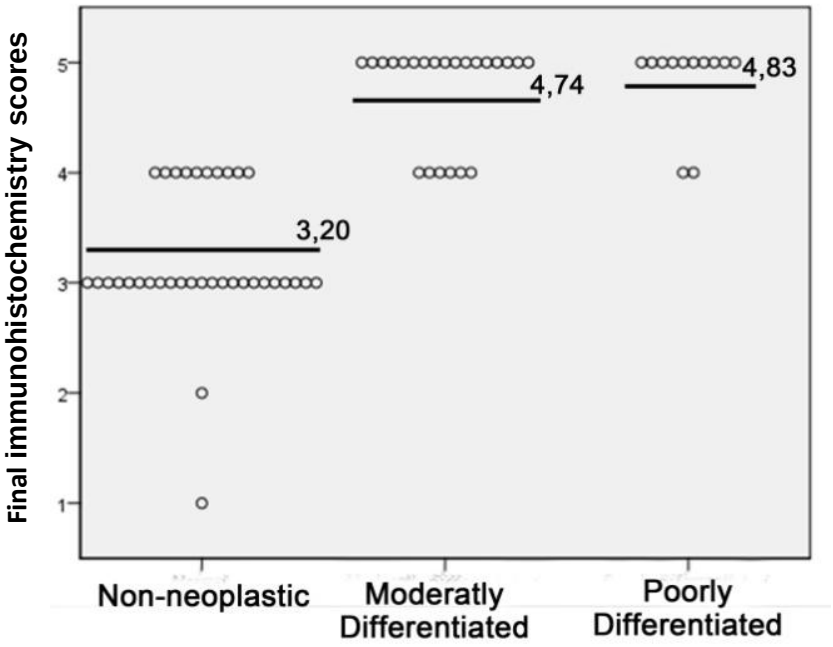


Figure 10 - Vimentin (Vim) expression in non-neoplastic, pancreatic ductal adenocarcinoma (PDA) and duodenum samples. Vim is expressed in nonepithelial cells (A) such as fibroblasts and inflammatory cells (B); Vim is abundantly expressed in tumoral desmoplastic stroma (C) to (F); This protein is also abundantly expressed in mesenchymal cells in duodenum, (G) in the plasma membrane of lymphocytes and in some scattered cells in duodenal epithelium (H);



Graph 5 - Frequencies of Vimentin final immunohistochemistry scores.. Mean value in non-neoplastic, moderately and poorly differentiated PDA samples is 3,20, 4,74 and 4,83, respectively.

3.3 Patient Characteristics

The patient characteristics are shown in table 7. From the 35 patients analyzed 19 (54,3%) were males and 16 (45,7%) were females. Ductal adenocarcinomas were located in the head of the pancreas (82,9% of the patients), in the body of the pancreas (2,9%) or in the ampulla of Vater region (14,3% of the patients). The pathologic stages after surgical resection were as follows: stage I (n = 4) and stage II (n = 31). Lymph node metastasis was identified in 71,4% of the patients. Perineural, vascular and intestinal invasion were positive in 60,0%, 34,3% and 60,0%, respectively. Histological examination revealed that ductal adenocarcinomas were predominantly moderately differentiated (65,7%) and were bigger than 2,5 cm (60,0%).

3.4 Relationship between AQP5, Ki-67, CK7, E-cad and Vim expression and Clinicopathological Features

The relationship between the expression level of AQP5, Ki-67, CK7, E-cad and Vim and the clinicopathological features is shown in table 7. The expression of these proteins was not associated with the parameters of gender, stage, tumor dimensions, lymph node metastasis, perineural, vascular and intestinal invasion. AQP5 and E-cad expression was found to be significantly correlated with tumor differentiation (AQP5, p=0,030; E-cad, p= <0,001): AQP5 and E-cad expression was higher in moderately differentiated adenocarcinomas than in poorly differentiated ones, while Ki-67 expression was significantly correlated with tumor location (p= 0,021).

Table 8: Relationship between AQP5, Ki-67, CK7, E-cad and Vim expression and clinicopathological features

Parameters	*n*	AQP5		P value	Ki-67		P value	CK7		P value	E-cad		P value	Vim		P value
		< 4	≥ 4		< 2	≥ 2		< 4	≥ 4		< 3	≥ 3		≥ 3	< 3	
Gender				0,284			0,347			0,937			0,486			0,424
Male	19 (54,3%)	2	14		8	8		2	14		3	13		13	3	
Female	16 (45,7%)	6	13		8	11		3	16		3	16		16	3	
Tumor diferrentiation				**0,030***			1,000			0,922			**<0,001***			0,685
Moderatly	23 (65,7%)	4	19		1	3		4	19		0	23		23	0	
Poorly	12 (34,3%)	4	8		15	16		1	11		6	6		6	6	
Stage				0,586			0,417			0,619			0,251			0,553
I	4 (11,4%)	0	4		6	8		1	3		0	4		4	0	
II	31 (88,6%)	8	23		10	11		4	27		6	25		25	6	
Dimensions				0,852			0,171			0,480			0,889			0,108
<2,5cm	14 (40,0%)	3	11		1	3		1	13		3	11		11	3	
≥2,5cm	21(60,0%)	5	16		15	16		4	17		3	18		18	3	
Tumor Location				0,899			**0,021***			0,124			0,106			0,466
Head	29 (82,9%)	8	21		16	13		3	26		6	23		23	6	
Body	1 (2,9%)	0	1		0	1		1	0		0	1		1	0	
Ampulla	5 (14,3%)	0	5		0	5		1	4		0	5		5	0	
Lymph node metastasis				0,921			0,944			0,137			0,540			0,390
Positive	25 (71,4%)	6	19		12	13		2	23		5	20		20	5	
Negative	10 (28,6%)	2	8		4	6		3	7		1	9		9	1	
Neuronal invasion				0,070			0,656			0,514			0,678			0,108
Positive	21 (60,0%)	8	13		11	10		3	18		3	18		18	3	
Negative	14 (40,0%)	0	14		5	9		2	12		3	11		11	3	
Vascular invasion				0,263			0,685			0,922			0,868			0,402
Positive	12 (34,3%)	5	7		5	7		1	11		2	10		10	2	
Negative	23 (65,7%)	3	20		11	12		4	19		4	19		19	4	
Intestinal invasion				0,209			1,000			0,871			1,000			1,000
Positive	21 (60,0%)	3	18		9	12		4	17		3	18		18	3	
Negative	14 (40,0%)	5	9		7	7		1	13		3	11		11	3	

4. Discussion

PC is one of the most intractable and mortal malignant tumors and its incidence is rapidly increasing worldwide [129]. Due to retroperitoneal location of the pancreas symptoms are rare in initial phases and when patients are diagnosed the disease is generally at advanced stage [129-131]. PDA is the most common type of PC (>85%) and the search for new biomarkers that prove to be useful to detect this disease in early stages will have a major impact in patients' lives. In the current study we evaluated the expression of AQP5, Ki-67, CK7, E-cad and Vim in 35 PDA patients and, to the best of our knowledge this is the first study to analyze the expression pattern of AQP5 and its clinical significance in PDA samples.

AQP5, a member of AQP family (AQP0-12), is a small integral transmembrane protein that plays a role in maintaining tissue water balance in different body locations [134]. Recent publications suggest that this isoform is up-regulated in a variety of systemic malignancies such as colon cancer [135, 136], breast cancer [137], lung cancer [138, 139], ovarian cancer [140], cervical cancer [141] and prostate cancer [142] being related with tumor recurrence, metastasis and poorer prognosis for patients by enhancing cancer cell proliferation, migration and survival [109, 135, 136, 138, 140, 142-148] through multiple pathways that are not yet fully understood. In tumors, the phosphorylation of AQP5 on Ser156 by PKA activates the RAS/MAPK pathway involved in cell proliferation and survival [94, 95]. AQP5 phosphorylation also promote the binding to the SH3 domain of adaptor molecules, such as c-Src [95], which is associated with EMT. AQP5 may additionally facilitate cancer cell motility due to its preferential polarization in the leading edge of migrating cells and by facilitating lamellipodium formation [100, 102, 105-107] at least, in lung cancer cells, partly through osmosensitive transcription factor NFAT5 regulation [149]. It is also known that AQP5 expressing cancer cells are resistant to apoptosis [101, 147, 148] and that AQP5 promoter region contains sequences for nuclear factor-κB (NF-κB) [127], suggesting that NF-κB may be involved in AQP5 transcription as well as it is involved in the transcription of anti-apoptotic genes [103, 104]. Recently, AQP5 was also associated with p38 MAPK pathway and with the expression of multidrug resistance proteins, responsible for colon cancer drug resistance [99].

Our results revealed that AQP5, Ki-67, CK7 and Vim are overexpressed in PDA samples while E-cad protein levels are down-regulated when compared with control samples. AQP5 overexpression was also found to be related with tumor differentiation. In fact, seems that moderately differentiated tumors, that recapitulate better the morphological ductal characteristics of normal pancreas, are higher AQP5-expressors than the poorly differentiated ones. These findings suggest that this protein may be used as a novel

biomarker for early stages of PDA. AQP5 overexpression in PDA may also be related with a better prognosis for patients and with less aggressive tumors which is supported by the lack of a relationship with the expression of the proliferation marker Ki-67. Moreover, immunohistochemistry revealed that in PDA cells AQP5 is expressed all over the plasma membrane and became to diffuse intracellularly. This pattern differs from the localized expression at the apical membrane of intercalated and intralobular ductal cells observed in normal pancreatic tissues [63]. Since tumorigenesis occurs in ductal epithelial cells it seems that AQP5 may play an important role in PDA progression and may represent a potential drug target for this disease. So it will be of great interest to study the AQP5 expression progression from normal cells through pancreatic intraepithelial neoplasias to invasive pancreatic tumors.

A positive correlation was also found between E-cad expression and tumor differentiation. Poorly differentiated tumors, that have a less cohesive morphology, being mainly constituted by isolated infiltrative cells and disrupted ducts, showed lower expression levels of this protein than moderately differentiated ones. On the other hand, Ki-67 expression, one of the most important markers that indicate active cellular proliferation, was significantly correlated with tumor location in the head of the pancreas and with Vim expression. No significant correlations were evident between the other analysed markers and other clinicopathological features.

Interestingly, we also found AQP5 strong expression in plasma membranes of some scattered cells in duodenal epithelium only when intestinal invasion was present. Although with weaker immunolabeling than for AQP5, these cells in duodenum also express CK7 and Vim all over the plasma membrane. Vim is a mesenchymal marker and CK7 is expressed in almost all cases of PDA [132]. It is certain that, CK7 is also expressed by primary adenocarcinomas of the small intestine [132], making it impossible to distinguish these type of tumors from PDA. However, primary tumors from small intestine are extremely rare [132] which makes this possibility really unlikely. Since in normal conditions duodenal epithelial cells do not express AQP5 [150], neither CK7 [132] or Vim [151], these observations support the idea that AQP5-duodenal expresser cells are, in fact, PDA migrating cells that escaped from the primary tumor and infiltrated the intestinal epithelium. Since these cells express higher protein levels of AQP5 (stronger immunolabeling) than CK7, this isoform seems to be a more sensitive biomarker to study intestinal infiltration by PDA cells.

5. Conclusions and perspectives

AQP5 overexpression in cancer cells and tumor tissues has been extensively reported. Consistent observations demonstrate that AQP5 is up-regulated in cancer, strongly suggesting its implication in carcinogenesis in different organs and systems. In this study we report, for the first time, that AQP5 is overexpressed in PDA and its overexpression is associated with a loss of polarity in ductal epithelial cells and with tumor histological differentiation/ grade, suggesting that AQP5 might be used as a novel biomarker to detect early disease stages/ less aggressive PDA tumors. Our preliminary findings also suggest that this isoform seems to be a more sensitive biomarker than CK7 to study intestinal infiltration by PDA cells.

Due to AQP5 involvement in cell migration, proliferation and adhesion in human cancers, this protein emerges as a promising drug target and its modulators as useful anti-tumor agents. Although the mechanisms by which AQP5 interferes with cell differentiation and participates in tumorigenesis are not completely clear, the information available supports its interaction with oncoproteins, such as RAS and c-Src and thus, its interplay with intracellular signalling transduction pathways. A similar mechanism may involve AQP5 in PDA tumorigenesis and this possibility requires further investigation.

The complexity of AQP5 therapeutic use is further illustrated by its involvement in both chemosensitivity and drug-resistance mechanisms in tumors. In addition, AQP5 differential expression among human tissues and tumors within different body systems, suggests the need of a tissue-targeted approach for anticancer treatment. The development of therapeutic strategies using AQP5 as drug target, however promising, needs a stronger basis on the molecular mechanisms responsible for its participation in tumour biology. In addition to AQP5 interaction with oncogenes, it is also possible that its function as a channel (transporting water or any signalling molecule such as hydrogen peroxide) is crucial for tumorigenesis. In this context, the modulation of AQP5 gating (open and closed channel) possibly through phosphorylation might be a powerful tool to prevent tumor development. In fact, the contrasting phosphorylation status between cancer and normal tissues suggests that the key role of this isoform in carcinogenesis is related with its phosphorylation rather than with its expression in cancer cells. Blocking the particular phosphorylation site of AQP5 in loop D may provide a unique opportunity in designing tumor-specific molecular inhibitors.

In conclusion, there is a great translational potential in AQP5-based therapeutics and diagnostics. In view of the wide range of cancer malignancies in which AQP5 is implicated, the potential of AQP5 as a biomarker for cancer detection and prognosis should be explored. Our results suggest that AQP5 might to be used as a novel biomarker for PDA aggressiveness allowing the detection of early disease stages. Due to its apparent role in

PDA tumorigenesis AQP5 may also represent a potential drug target for this disease. However, further pathophysiological investigation is required to establish AQP5 as a PDA drug target and as a biomarker for PDA detection and follow up.

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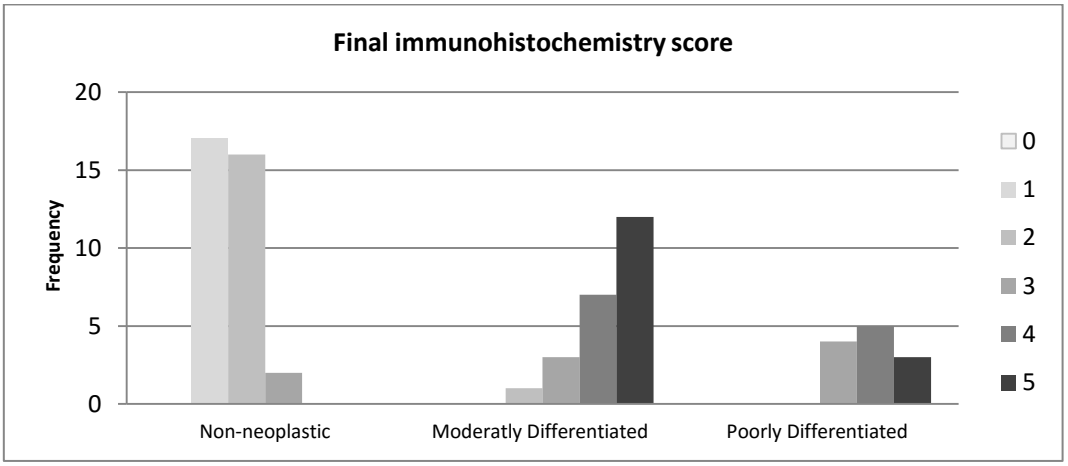
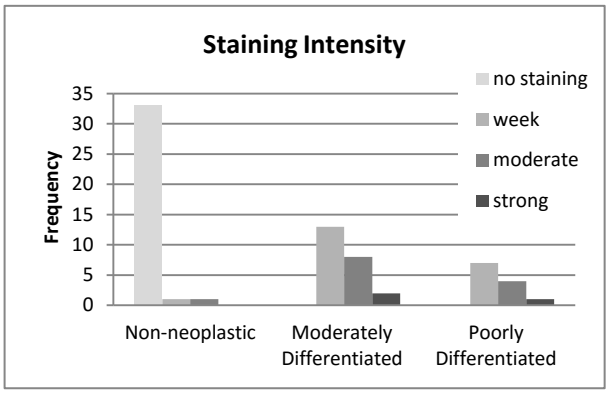
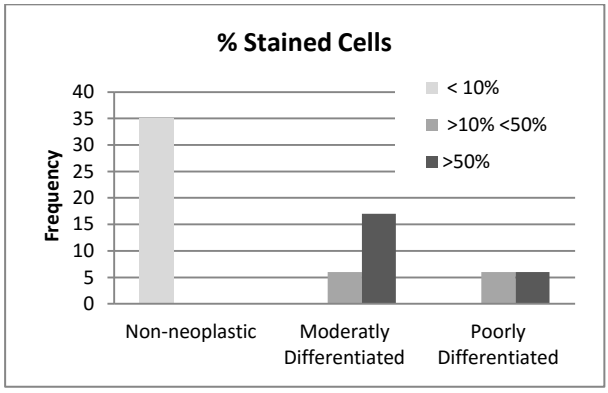
Appendix



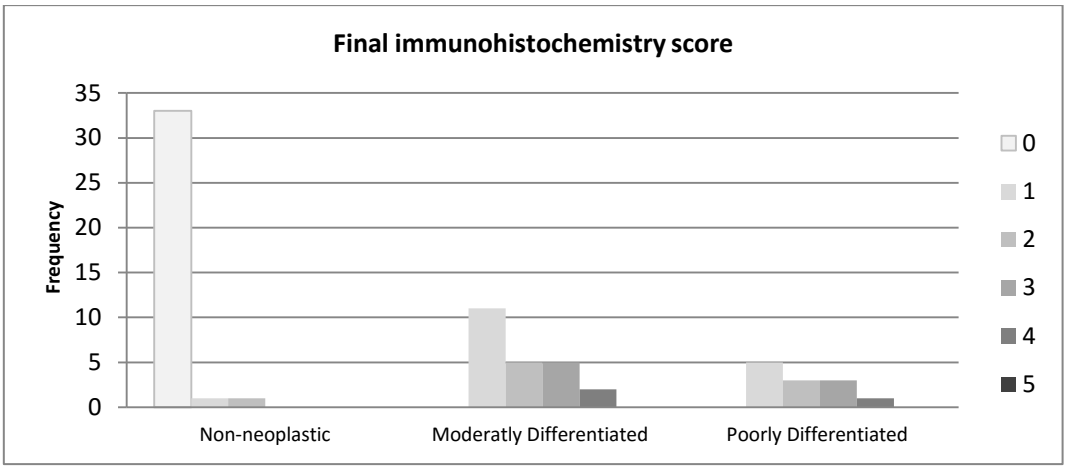
Graph 6 - Frequencies of Aquaporin 5 final immunohistochemistry scores. Of the 35 non-neoplastic samples 17 had 1 as final score, 16 had 2 and 2 cases had a final score of 3; of the 23 moderately differentiated adenocarcinomas 1 case was evaluated with a final score of 2, 3 with a final score of 3, 7 with a final score of 4 and 12 with a final score of 5; of the 12 poorly differentiated adenocarcinomas 4 cases ware evaluated with a final score of 3, 5 with a final score of 4 and 3 with a final score of 5.



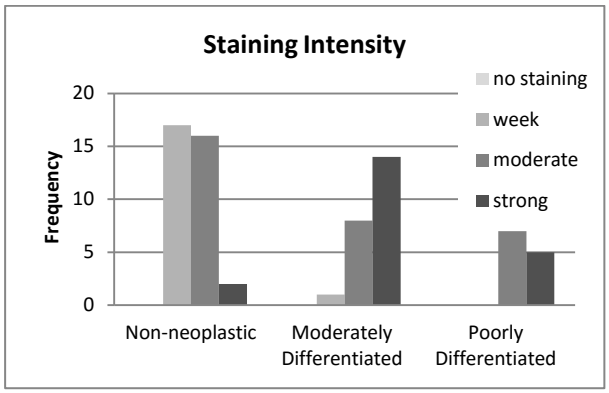
Graph 7 - Aquaporin 5 staining intensity: score frequencies. Of the 35 non-neoplastic samples 17 showed week staining, 16 moderate staining and 2 strong staining; 1 moderately differentiated sample showed week staining, 8 moderate staining and 14 strong staining; 7 poorly differentiated samples showed moderate staining and 5 strong staining.



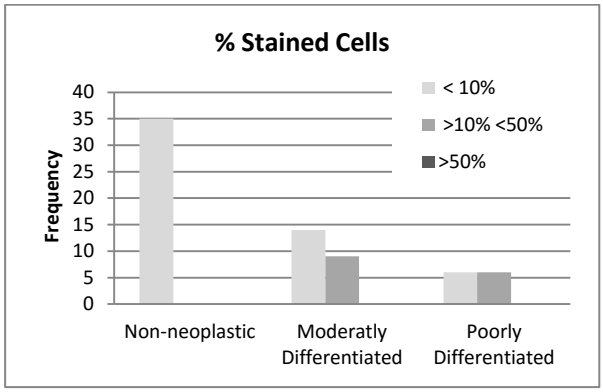
Graph 8 - Percentage of stained cells with Aquaporin 5 (AQP5): score frequencies. Observed stained cells were less than 10% in all non-neoplastic samples; 6 moderately differentiated samples presented 10% to 50% stained cells and 17 more than 50%; 6 poorly differentiated samples presented 10% to 50% stained cells and the other 6 more than 50%.



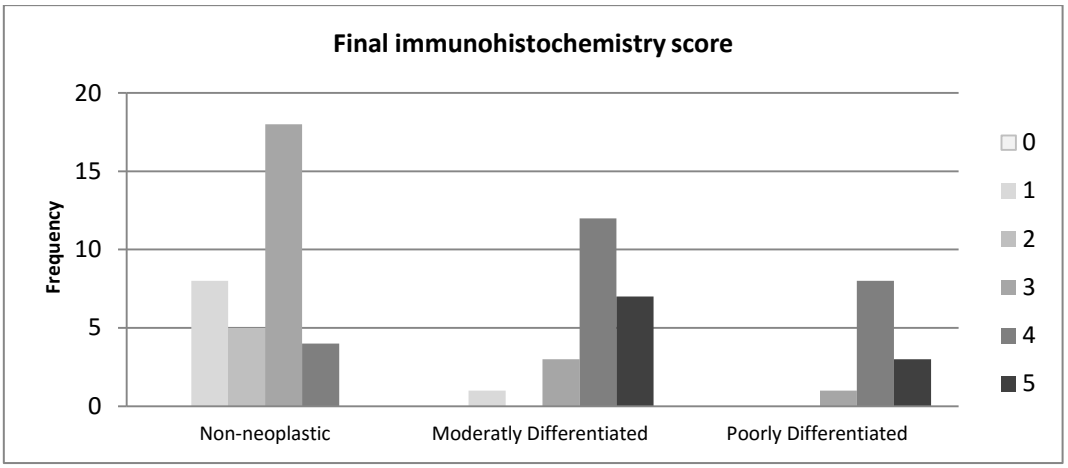
Graph 9 - Frequencies of Ki-67 final immunohistochemistry scores. Of the 35 non-neoplastic samples 33 had 0 as final score, 1 had 1 and the another case had a final score of 2; of the 23 moderately differentiated adenocarcinomas 11 cases were evaluated with a final score of 1, 5 with a final score of 2, 5 with a final score of 3 and 2 with a final score of 4; of the 12 poorly differentiated adenocarcinomas 5 cases ware evaluated with a final score of 1, 3 with a final score of 2, 3 with a final score of 3 and 1 with a final score of 4.



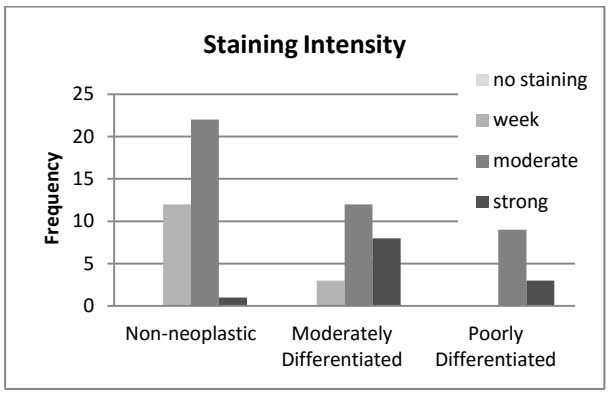
Graph 10 - Ki-67 staining intensity: score frequencies. Of the 35 non-neoplastic samples 33 showed no staining, 1 week staining and 1 moderate staining; 13 moderately differentiated samples showed week staining, 8 moderate staining and 2 strong staining; 7 poorly differentiated samples showed week staining, 4 moderate staining and 1 showed strong staining.



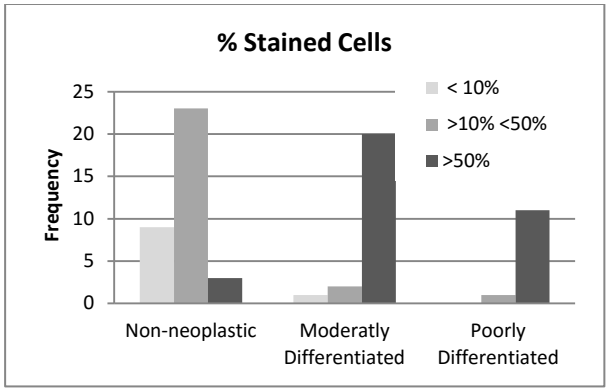
Graph 11 - Percentage of stained cells with Ki-67: score frequencies. Observed stained cells were less than 10% in all non-neoplastic samples, in 14 moderately differentiated samples and in 6 poorly differentiated samples; 9 moderately differentiated samples and 6 poorly differentiated samples presented 10% to 50% stained cells.



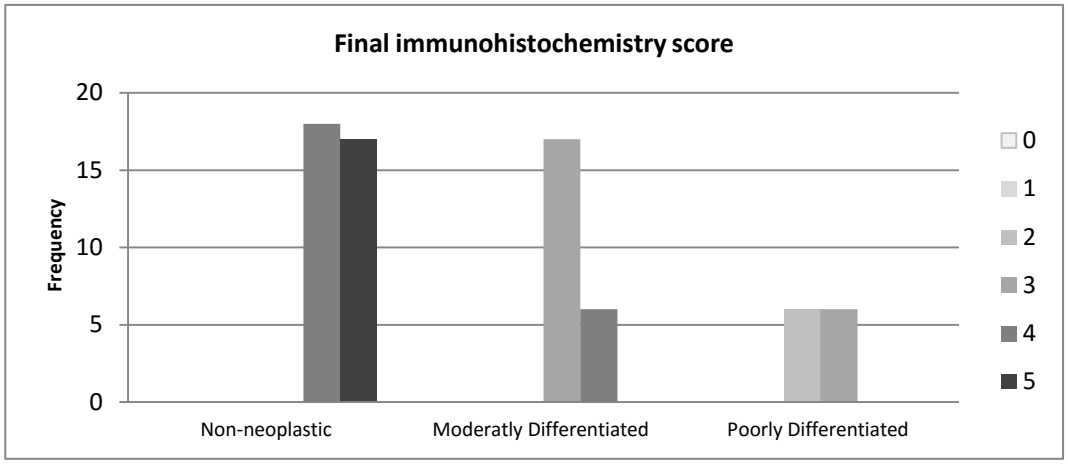
Graph 12 - Frequencies of Cytokeratin 7 final immunohistochemistry scores. Of the 35 non-neoplastic samples 8 had 1 as final score, 5 had 2, 18 had 3 and 4 cases had a final score of 4; of the 23 moderately differentiated adenocarcinomas 1 case were evaluated with a final score of 1, 3 with a final score of 3, 12 with a final score of 4 and 7 with a final score of 5; of the 12 poorly differentiated adenocarcinomas 1 case ware evaluated with a final score of 3, 8 with a final score of 4 and 3 with a final score of 5.



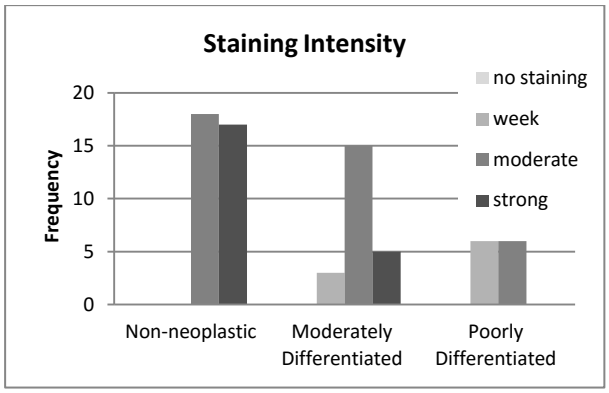
Graph 13 - Cytokeratin 7 staining intensity: score frequencies. Of the 35 non-neoplastic samples 12 showed week staining, 22 moderate staining and 1 strong staining; 3 moderately differentiated samples showed week staining, 12 moderate staining and 8 strong staining; 9 poorly differentiated samples showed moderate staining and 3 showed strong staining.



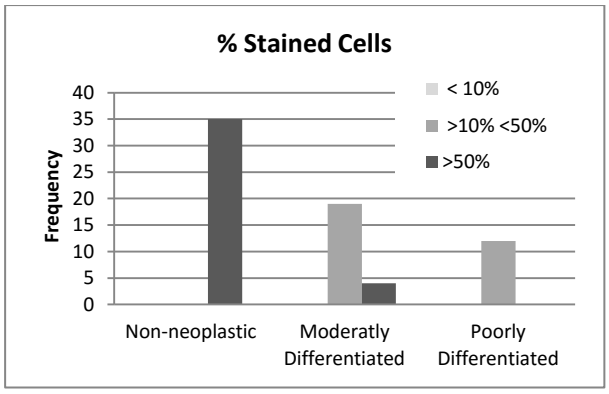
Graph 14 - Percentage of stained cells with Cytokeratin 7: score frequencies. Less than 10% of cells were stained in 9 non-neoplastic samples and in 1 moderately differentiated sample; 10% to 50% stained cells were observed in 23 non-neoplastic samples, 9 moderately differentiated samples and in 6 poorly differentiated samples; 3 non-neoplastic samples, 20 moderately differentiated samples and 10 poorly differentiated samples had more than 50% stained cells.



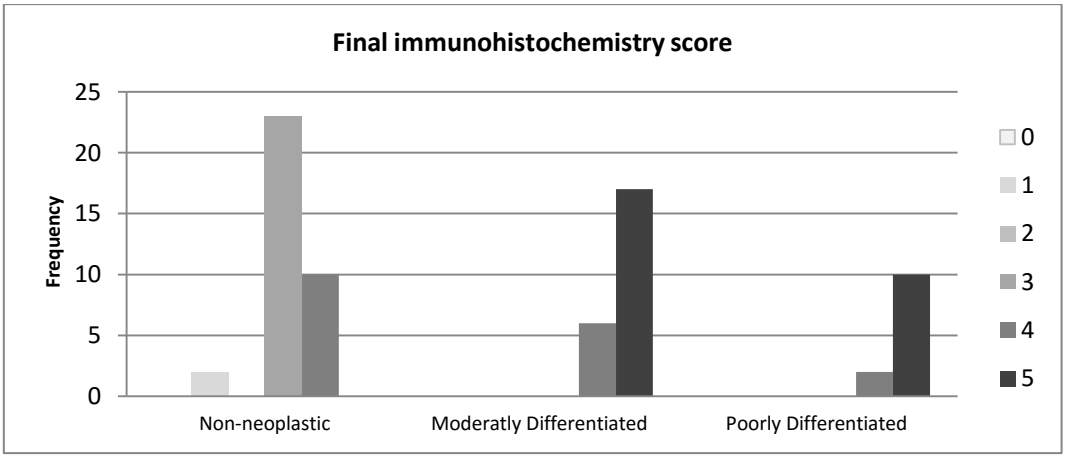
Graph 15 - Frequencies of E-Cadherin final immunohistochemistry scores. Of the 35 non-neoplastic samples 18 had 4 as final score and 17 had a final score of 5; of the 23 moderately differentiated adenocarcinomas 17 cases were evaluated with a final score of 3 and 6 with a final score of 4; of the 12 poorly differentiated adenocarcinomas 6 cases ware evaluated with a final score of 2 and the other 6 with a final score of 3. Mean value in non-neoplastic, moderately and poorly differentiated PDA samples is 4,49, 3,26 and 2,50, respectively.



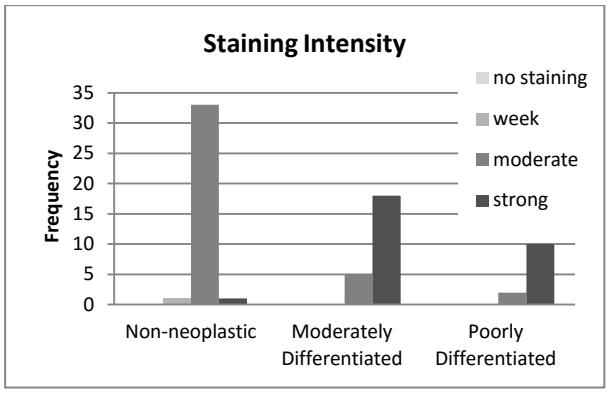
Graph 16 - E-cadherin staining intensity: score frequencies. Of the 35 non-neoplastic samples 18 showed moderate staining and 17 strong staining; 3 moderately differentiated samples showed week staining, 15 moderate staining and 5 strong staining; 6 poorly differentiated samples showed week staining and 6 showed moderate staining.



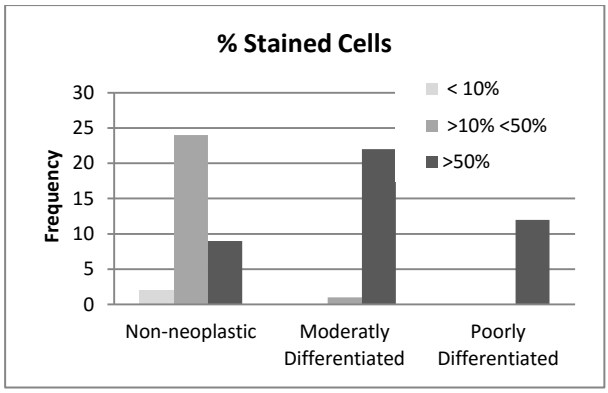
Graph 17 - Percentage of stained cells with E-cadherin: score frequencies. Observed stained cells were more than 50% in all non-neoplastic samples and in 4 moderately differentiated samples; 10% to 50% stained cells were observed in 19 moderately differentiated samples and in all of poorly differentiated samples.



Graph 18 - Frequencies of Vimentin final immunohistochemistry scores. Of the 35 non-neoplastic samples 2 had 1 as final score; 23 had 3 and 10 had a final score of 4; of the 23 moderately differentiated adenocarcinomas 6 cases were evaluated with a final score of 4 and 17 with a final score of 5; of the 12 poorly differentiated adenocarcinomas 2 cases were evaluated with a final score of 4 and 10 with a final score of 5.



Graph 19 - Vimentin staining intensity: score frequencies. Of the 35 non-neoplastic samples 1 case showed week staining, 33 showed moderate staining and 1 strong staining; 5 moderately differentiated samples showed moderate staining and 18 strong staining; 2 poorly differentiated samples showed moderate staining and 10 strong moderate staining.



Graph 20 – Percentage of stained cells with Vimentin: score frequencies. Observed stained cells were less than 10% in 2 non-neoplastic samples; 10% to 50% stained cells were observed in 24 non-neoplastic samples and in 1 moderately differentiated sample; more than 50% stained cells were observed in 9 non-neoplastic samples, in 22 moderately differentiated samples and in all of poorly differentiated samples.