Elisabete Sofia Antunes Dias Nobre Fonseca

Licenciada em Bioquímica

Aquaporins as Biomarkers for Pancreatic Cancer

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

Orientadora: Prof. Doutora Graça Soveral, Professora Catedrática,
Faculdade de Farmácia, Universidade de Lisboa.

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Elisabete Fonseca

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“Remember, remember, this is now, and now, and now.
Live it, feel it, cling to it.
I want to become acutely aware of all I’ve taken for granted.”
SYLVIA PLATH

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Gostaria de começar por agradecer à professora Graça Soveral, por me ter proporcionado a oportunidade de trabalhar neste projeto, pelo qual me apaixonei no primeiro dia. Obrigada por me ter acolhido e tratado tão bem durante este último ano. Obrigada por me deixar aprender consigo. A ambas as Inês e à Catarina por tudo o que me ensinaram, especialmente sobre a pessoa que quero ser. À Joana por ter sido a minha companheira nesta aventura, não teria conseguido sem ti!

À minha família. Mãe, pai, Neuza, Nicolas, Melanie, Jorge e Leandro. São a luz que me guia e a certeza que me prende ao chão. Obrigada por me amarem mesmo quando não mereço.

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Abstract

By 2030, pancreatic cancer is anticipated to overtake lung cancer as the second leading cause of cancer-related death. Surgery is now the primary treatment option for treating pancreatic cancer, but it's crucial to remember that only a very small proportion of patients have a resectable tumour at the time of diagnosis. An early pancreatic cancer diagnosis is still difficult due to the absence of distinguishing symptoms and distinct clinical indications.

Aquaporins (AQPs) are a family of water channel proteins. Their function of facilitating water and glycerol, among other solutes, has been linked to carcinogenesis while being overexpressed in various cancer types. Therefore, aquaporins' expression has been associated with tumour cell motility, tumour proliferation, and the potential for metastasis. Several signalling pathways, including JNK/ERK/p38 MAPK, P13K/AKT/mTOR, and Wnt/β-catenin, growth factors, and even epithelial-mesenchymal transition, are also correlated to the activity and regulation of AQPs.

The global aim of this work is to determine aquaporins' expression in pancreatic cancer, their interplay with pathways involved in tumorigenesis and to validate aquaporins as possible prognosis and/or diagnosis biomarkers in pancreatic cancer.

25-paired healthy and tumour pancreatic tissues in different stages of differentiation were analysed by real-time quantitative polymerase chain reaction for relative expression of aquaporin-1 (AQP1), aquaporin-3 (AQP3), aquaporin-5 (AQP5), aquaporin-9 (AQP9), e-cadherin (Ecad), vimentin (Vim), epidermal growth factor receptor (EGFR), extracellular signal-regulated protein kinase-1 (ERK1), extracellular signal-regulated protein kinase-2 (ERK2), proto-oncogene Jun (c-Jun), and proto-oncogene Fos (c-Fos), both transcription factors subunits of activator protein 1 (AP-1). These results were analised according to variables such as age, gender, invasiveness grade, and aggressiveness grade.

Aquaporins' mRNA was detected in both healthy and tumour tissues. Ecad and Vim mRNA levels were higher in high-invasive grade tumours in female patients. ERK1 mRNA level was increased in low-invasive grade tumours from male patients. c-Jun and AQP3 were both overexpressed in low-invasive grade tumours. AQP3 and AQP9 correlated positively with c-Jun in tumour tissues, while AQP5 correlated with EGFR and Ecad.

AQP9 was for the first time detected in pancreatic healthy and tumour tissue. AQP3 has been identified as a possible biomarker for the invasiveness grade. This aquaporin is also correlated in tumour tissues with c-Jun, stating a possible involvement in cell proliferation and cell survival. AQP3 and AQP9 are directly involved in JNK MAPK pathway, while AQP5 may be indirectly involved in ERK MAPK pathway and epithelial-mesenchymal transition.

Keywords: aquaporins; pancreatic cancer; biomarker; ERK MAPK signalling pathway; epithelial-mesenchymal transition.

Resumo

Em 2030, o cancro do pâncreas deverá ultrapassar o cancro do pulmão como a segunda principal causa de morte relacionada com o cancro. A cirurgia é ainda a principal opção de tratamento para o cancro do pâncreas, mas é crucial relembrar que apenas uma proporção muito pequena de pacientes tem um tumor ressecável no momento do diagnóstico. O diagnóstico precoce do cancro de pâncreas ainda é difícil devido à ausência de sintomas evidentes e indicações clínicas específicas.

As Aquaporinas (AQPs) são uma família de canais de transporte de água. A sua função de transporte facilitado de água e glicerol, entre outros solutos, tem sido associada com carcinogénese, estando sobre expressas em vários tipos de cancro. Em consequência, a sua expressão tem sido associada com a mobilidade células tumorais, proliferação celular e com o potencial de metástase. Várias vias de sinalização, incluindo a JNK/ERK/p38 MAPK, P13K/AKT/mTOR e Wnt/β-catenina, bem como fatores de crescimento e até a transição epitélio-mesenquimal estão também correlacionadas com a atividade e regulação das AQPs.

O objetivo global deste trabalho é determinar a expressão das aquaporinas no cancro do pâncreas, a interação com vias envolvidas na tumorigenese e validar as aquaporinas como possíveis biomarcadores de prognóstico e/ou diagnóstico no cancro do pâncreas

Neste estudo, 25 pares de tecidos saudáveis e tumorais de pâncreas em diferentes estágios de diferenciação foram analisados por reação em cadeia da polimerase quantitativa em tempo real para a expressão relativa de aquaporina-1 (AQP1), aquaporina-3 (AQP3), aquaporina-5 (AQP5), aquaporina-9 (AQP9), E-caderina (Ecad), vimentina (Vim), recetor do fator de crescimento epidérmico (EGFR), proteína quinase-1 regulada por sinal extracelular (ERK1), proteína quinase-2 regulada por sinal extracelular (ERK2), proto-oncogene Jun (c-Jun) e proto-oncogene Fos (c-Fos), sendo ambos fatores de transcrição da proteína ativadora 1 (AP-1). Estes resultados foram analisados de acordo com variáveis como a idade, o sexo, o grau de invasão e o grau de agressividade.

O mRNA das aquaporinas foi detetado em tecidos saudáveis e tumorais. Os níveis de mRNA da Ecad e Vim foram mais elevados em tumores de alto grau invasivo em pacientes do sexo feminino. O nível de mRNA da ERK1 está aumentado em tumores de baixo grau invasivo de pacientes do sexo masculino. c-Jun e AQP3 estão ambas sobre expressas em tumores de baixo grau invasivo. AQP3 e AQP9 correlacionaram-se positivamente com a c-Jun em tecidos tumorais, enquanto AQP5 correlacionou-se com EGFR e Ecad.

A AQP9 foi pela primeira vez identificada em tecidos saudáveis e tumorais de pâncreas, enquanto a AQP3 foi identificada como um possível biomarcador para o grau de invasão, sendo correlacionada em tecidos tumorais com a c-Jun, descrevendo o possível envolvimento desta aquaporina com a proliferação e sobrevivência celular. AQP3 e AQP9 estão diretamente envolvidos com a via JNK MAPK, enquanto AQP5 pode estar indiretamente envolvida com a via ERK MAPK e a transição epitélio-mesenquimal.

Termos chave: aquaporinas; cancro do pâncreas; biomarcador; via de sinalização ERK MAPK; transição epitélio-mesenquimal.

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List of Abbreviations

ACTB - β-actin

AKT – Serine/Threonine Kinase

AP-1 – Activator protein 1

AQP(s) – Aquaporin(s)

AQP0 - AQP12 - Aquaporin 0 to Aquaporin 12

C – Carboxyl-termini

cDNA – Completary DNA

CE – Cairicoside E

c-Fos – Fos Proto-oncogene AP-1 Transcription factor subunit

c-Jun – Jun Proto-oncogene AP-1 Transcription factor subunit

CRC – Colorectal cancer

CT – Threshold Cycle

DEPC-treated water – Diehtylpyrocarbonate-treated water

DNA – Deoxyribonucleic acid

Ecad - E-cadherin

EGF – Epidermal Growth Factor

EGFR – Epidermal growth factor receptor

ELK1 – ETS Transcription Factor

EMT – Epithelial-mesenchymal transition

ERK1 – Extracellular signal-regulated protein kinase-1

ERK2 – Extracellular signal-regulated protein kinase-2

HER2 – Human epidermal growth factor receptor 2

HPRT1 – Hypoxanthine-guanine phosphoribosyltransferase 1

JNK – c-Jun terminal kinase

MAPK – Mitogen-activated protein kinase

MEK – Mitogen-activated protein kinase kinase

MIP – Major intrinsic protein

mRNA – Messenger RNA

mTOR – Mammalian target of rapamycin

N – Amino-termini

NPA – asparagine-proline-alanine

OS – Overall Survival

P13K – Phosphatidylinositol 3-kinase

PDAC – Pacreatic ductal adenocarcinoma

Raf – Rapidly Accelerated Fibrosarcoma

RNA – Ribonucleic Acid

RTKs – Receptor Tyrosine Kinases

RT qPCR – Real Time Quantitative Polimerase Chain Reaction

ssRNA – Single stranded RNA

TNM – Classification system; T refers to the size and extent of the main tumour; N refers to the number of nearby lymph nodes that have cancer; M refers to whether the cancer has metastasized.

Vim - Vimentin

1. Introduction

1.1. Discovering Aquaporins

The physiological function of tissues and organs is dependent on cells' capacity to exchange water rapidly between the external environment and their cytoplasm, maintaining a constant volume. This will impact the cells' response to metabolic demands and the secretion and/or absorption of water and other fluids (Brown, 2017).

Studies of water transport were first initiated in tissues more water permeable than other tissues, such as the skin and bladder of certain amphibians, in 1965 (Ussing, 1965). In red blood cells, the studies showed water transport with low Arrhenius activation energy, implying the presence of a pore in the membrane (Solomon, 1968). Electron microscopy allowed the observation of structures which were presumed to be water channels (Frøkiær et al., 2009). It was also observed that under conditions when water permeability increased, these proteins would also become more numerous (Kachadorian et al., 2000).

Since then, the molecular identity and structure of these water channels were investigated, with many different strategies (Frøkiær et al., 2009). It was not until 1987 that the first aquaporin was identified during the identification search of the Rh blood group antigens (Agre et al., 1987; Saboori et al., 1988). One of the Rh antigens was isolated and found to be about 32 kDa. When trying to detect the protein, another protein of 28 kDa was found and was initially thought to be a proteolytic fragment of the 32 kDa protein. However, the 28 kDa protein's specific antibody did not bind to the 32 kDa protein; the characterization of these proteins determined that they were not related (Denker et al., 1988). With further research, the 28 kDa protein was discovered to have several physical properties of a membrane channel. Thus, it was named a channel-like integral protein of 28 kDa (CHIP28) (Smith & Agre, 1991).

The expression of CHIP28 in *Xenopus laevis* oocytes allowed its first identification as a water channel (Preston et al., 1992). Oocytes are very useful and therefore used in the study of water transport proteins, since oocytes have low water permeability (Frøkiær et al., 2009). It was observed that oocytes expressing CHIP28 swelled and exploded when in dilute Modified Barth's solution, while for the control oocytes swelling was insignificant. Furthermore, the osmotic water permeability coefficient of CHIP28-expressing oocytes was 20-fold higher than the control. Regarding the Arrhenius activation energy, CHIP28 oocytes had close values to the water transport in native membranes, and lower than that of the control.

Later, CHIP28 homologs were identified, and the name was changed to Aquaporin 1 (AQP1), following the constitution of Aquaporins (AQPs) as a family of water channels (Agre et al., 1993).

The studies on AQP1 characterization in red blood cells predicted the functional structure as a homotetramer (Smith & Agre, 1991). A similar AQP1 homolog was also found in microorganisms, plants, and mammals. The observations made by the time on the lens fibre major intrinsic proteins (MIP), now named aquaporin 0 (AQP0), allowed the prediction of the six membrane-spanning domains (Gorin et al., 1984), which were later confirmed for AQP1 and the following aquaporins.

In subsequent studies, some member of the aquaporin family were found to transport not only water, but glycerol, urea, anions, ammonia, arsenite, antimonite, carbon dioxide, , hydrogen peroxide and nitric oxide gases (Frøkiær et al., 2009).

1.2. Structure and Function

AQPs are tetrameric hydrophobic intrinsic membrane proteins (Verkman & Mitra, 2000), forming a family of water channels proteins, with 13 isoforms detected in mammals (AQP0-AQP12) (Verkman, 2012), also expressed in archaea, eubacteria, fungi, and plants with a family of 35 isoforms (Kruse et al., 2006; Maure et al., 2008).

The monomers structures are small, ranging from 24 to 34 kDa (Kruse et al., 2006; Verkman & Mitra, 2000), containing six nonpolar regions forming six transmembrane helices (H1-H6) connected by five loops (A-E) (Verkman & Mitra, 2000), as illustrated in figure 1 (Wittekindt & Dietl, 2019). Of these, loops A, C, and E are extracellular and loops B and D are intracellular. Loop B and E form a seventh transmembrane domain, responsible for the aqueous pathway through the proteinaceous pore. Both loops enter the membrane and have a conversed signature motif, two asparagine-proline-alanine (NPA), that are oriented 180 degrees to each other and form a pathway. Furthermore, both amino- (N) and carboxyl-termini (C) are cytoplasmically oriented (Verkman & Mitra, 2000). Aquaporins can also have consensus sequences for N-linked glycosylation, being monoglycosylated in native tissues. Nevertheless, glycosylation has not shown any involvement in aquaporin function or membrane targeting (Van Hoek et al., 1995).

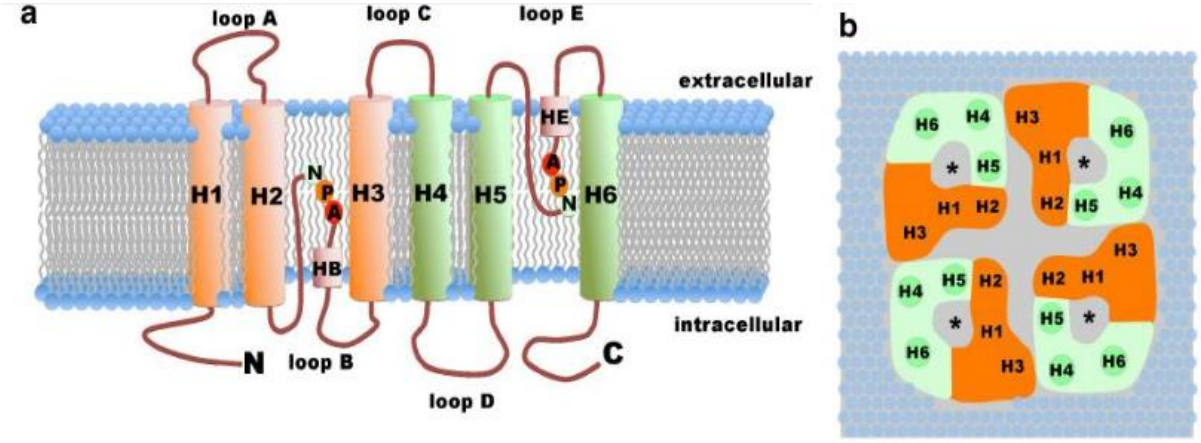

Figure 1 - An illustration of an aquaporin. a Aquaporins have six transmembrane segments and cytosolic N- and C-terminal domains (H1 to H6). Loops A, C, and E, which are extracellular, connect H1 with H2, H3 with H4, and H5 with H6. Loops B and D, which are intracellular loops, link H2 with H3, and H5 with H6. The NPA motivations are found in loop B and loop E. b View of an AQP tetramer's top extracellular surface taken from above. Shown is the configuration of the transmembrane segments H1 to H3 (in orange) and H4 to H6 (in green). Each AQP monomer's water pore is indicated by an asterisk. Retrieved and dapted from Wittekindt & Dietl (2019).

Aquaporins were believed to only facilitate water transport. However, with further research and the growing interest in AQPs, within the multiple isoforms, subgroups were defined depending on the transport capacity (Abrami et al., 1996; Echevarría et al., 1996; Hasegawa et al., 1994; Henzler & Steudle, 2000; Ishibashi et al., 1997; Koyama et al., 1998; M. D. Lee et al., 1996; K. Liu et al., 2006; B. Yang & Verkman, 1997): classical aquaporins, which are considered strict water channels; aquaglyceroporins, which can facilitate glycerol transport and of other larger molecules, besides water; unorthodox or super aquaporins, with still undefined transport capability, and low homology to the other groups, being absent in plants, bacteria and fungi (Ishibashi, 2006; Ishibashi et al., 2014); more recently, peroxiporins, which can also transport hydrogen peroxide. The family subgroups are summarized in table 1.

Table 1 - Classes of Mammalian Aquaporins.

Mammalian Aquaporins Classes	Permeability	Aquaporins
Classical Aquaporins	Water	AQP0, AQP1, AQP2, AQP4, AQP5, AQP8
Aquaglyceroporins	Water, urea, glycerol, small solutes	AQP3, AQP6, AQP7, AQP9, AQP10
Unorthodox Aquaporins	Water, glycerol (AQP11) Uncertain (AQP12)	AQP11, AQP12
Peroxiporins	Hydrogen Peroxide	AQP1, AQP3, AQP5, AQP8, AQP9, AQP11

The most impressive aspect of AQP channels is their excellent selectivity and efficiency on water or glycerol permeation while excluding ions and protons (Murata et al., 2000). Aquaporins have been identified in organs and tissues involved in fluid transporting, e.g., liver, lungs, and kidneys, but also in non-absorbing or non-excretory organs and tissues, e.g., brain, and fat tissue (Ishibashi et al., 2009). In consequence, AQPs are involved in osmoregulation and water homeostasis in organs involved in fluid transport, and energy metabolism regulation in non-fluid transport organs, by controlling glycerol content (Kortenoeven & Fenton, 2014; Törnroth-Horsefield et al., 2010). This implies AQPs' role in triggering cell mechanisms such as cell proliferation, migration, skin hydration, adipocyte and fat metabolism, brain edema, and carcinogenesis (Hara-Chikuma & Verkman, 2006; Ribatti et al., 2014; Rodríguez et al., 2011; Verkman et al., 2014). The distribution of aquaporins in human tissues and organs are illustrated in figure 2. (Azad et al., 2021).

AQPs have been found involved in several pathological conditions including cancer. Their aberrant expression was reported in multiple cancer types, brain swelling, neuroexcitation, glaucoma, skin hydration, obesity, diabetes, and others (Verkman, 2012). For these reasons, aquaporins are described as innovative therapeutic molecular targets or possible new prognostic biomarkers for different diseases.

Thus, understanding the regulatory mechanisms involved in AQPs activity is imperative. The current literature explains the regulatory mechanisms of AQPs' trafficking or gating properties, and channel abundance at the plasma cellular membrane. AQPs' regulation was described in various cell systems, being influenced by factors such as phosphorylation, temperature, pH, pressure, solute gradients, and membrane tension (Chaumont et al., 2005; Leitão et al., 2012, 2014; Maurel, 2007; Ozu et al., 2013; Soveral et al., 1997, 2008; Törnroth-Horsefield et al., 2010). AQPs' activity and regulation are also associated with multiple signalling pathways, c-Jun N-termina kinase (JNK)/ induced extracellular signal-regulated kinase (ERK)/ p38 mitogen-activated protein kinase (MAPK), phosphatidulinositol 3-kinase (P13K)/ serine/threonine kinase (AKT)/ mammalian target of rapamycin (mTOR), wingless/integrated (Wnt)/β-catenin, and others (Nito et al., 2012; Xu et al., 2011; Zhou et al., 2021). The enlightenment on these regulatory mechanisms is necessary to understand the impact of aquaporins' activity in healthy and diseased tissues, as well as to develop therapeutic approaches, and prognosis strategies.

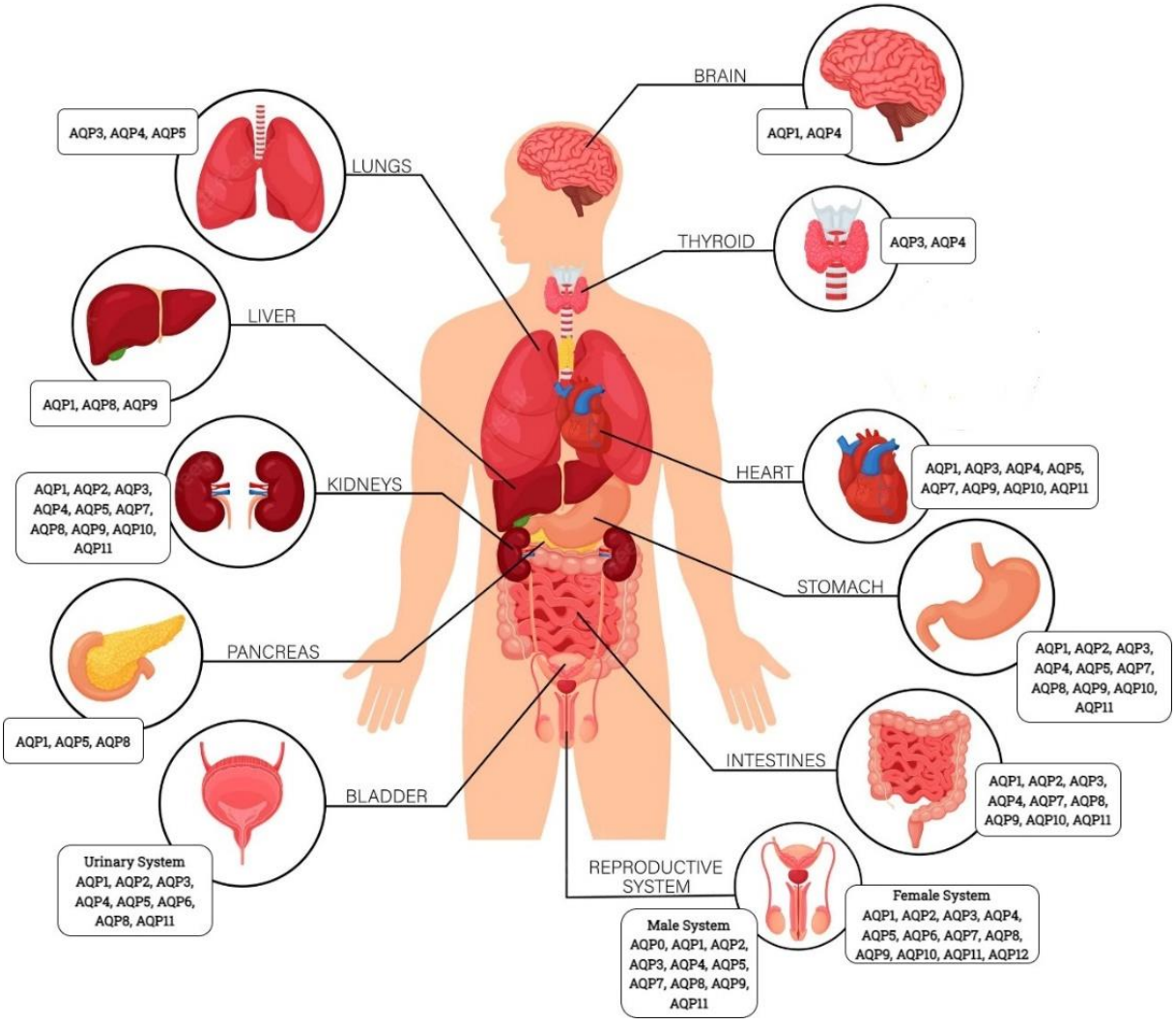

Figure 2 - Distribution of aquaporins through the different organs and reproductive system in humans. Adapted from Azad et. al (2021).

1.3. Aquaporins in Cancer

Several studies have reported AQPs overexpression in tumour tissues, suggesting a role for AQPs in cancer (Papadopoulos & Saadoun, 2015). Furthermore, there is usually a strong correlation between tumour grade in brain tumours and human glioblastoma and the level of AQP expression (Saadoun et al., 2002; Warth et al., 2004). Further research has found that in some tumour types the AQP expression is reduced compared to their originating cell, however only when considering individual values for each AQP; when several AQPs are considered, the overall AQPs expression increases (Papadopoulos & Saadoun, 2015).

AQPs expression allows water to rapidly infiltrate the growing tumour mass (Nico & Ribatti, 2010), and has been associated with tumour expansion (Marmarou, 2007), tumour cell migration, metastatic potential, and tumour-associated oedema (Hoque et al., 2006). Although AQPs' role of maintaining tissue water balance is the principal function, other roles appointed to AQPs, especially regarding tumour cells in facilitating cell migration, proliferation and adhesion, as illustrated in figure 3.

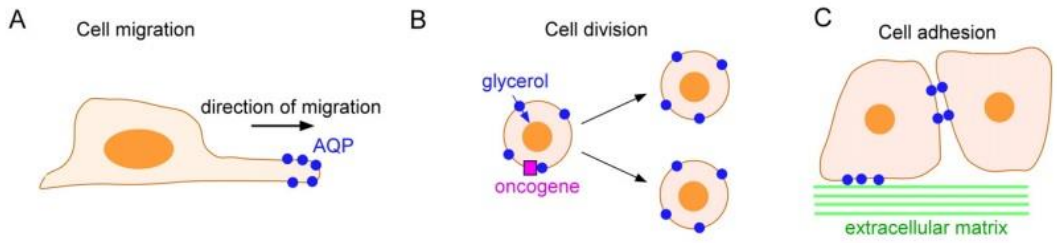

Figure 3 - Aquaporins' roles in cancer. A. The development of the lamellipodium and stabilization by actin polymerization is aided by AQP's polarization to the cell's leading edge and water fluxes facilitation, triggered by an increase in local osmolarity due to transmembrane ion fluxes. B. The uptake of permeants such as glycerol and hydrogen peroxide is facilitated by AQPs, and they may also interact with oncoproteins to initiate intracellular signalling cascades that stimulate the transcription of genes involved in the proliferation of tumour cells. C. Binding of AQP0-AQP0 and/or AQP4-AQP4 promotes cell-cell adhesion, while AQPs also might make cells more adherent to the extracellular matrix. Retrieved and adapted from Papadopoulos (2015).

Different cancers share the underlying characteristic of tumour cell migration, which promotes tumour cell infiltration into surrounding tissue and the propagation of metastatic disease. AQPs' function in facilitating cell migration has been studied in neutrophils (AQP9) (Loitto et al., 2001), aortic endothelial cells (AQP1) (Saadoun, Papadopoulos, Hara-Chikuma, et al., 2005), and astrocytes (AQP4) (Saadoun, Papadopoulos, Watanabe, et al., 2005), and the overall conclusion is that the expression of AQP promotes cell migration in response to chemotactic stimulation. While the mechanism is still unknown, evidence uncovered the water input into migrating cell's leading edge, enhancing lamellipodium development. Lamellipodium is a flattened protrusion localized in the leading end of a migrating cell, indispensable for cell motility (Bisi et al., 2013). AQPs polarize to the leading end of migrating cells, which is consistent with the concept of AQPs facilitating the formation of the lamellipodium (Papadopoulos & Saadoun, 2015). It was also proposed that water permeation, mediated by AQPs, may contribute to a net inflow of water and ions at the leading edge of the cell and a net outflow of water and ions at the trailing edge, resulting in a net cell displacement (Stroka et al., 2014). Nevertheless, the lack of AQPs does not inhibit cell migration but makes migratory response to a chemotactic stimulus less effective (Papadopoulos & Saadoun, 2015).

AQP1 particularly has been associated with tumour growth, metastasis, and local infiltration, by enhancing angiogenesis and cell migration (Hu et al., 2006). Extravasation was more common in 4T1 breast cancer cells and B16F10 melanoma cells that had been transfected to produce AQP1. Compared to tumour cells lacking AQP1, the resulting lung tumours were more widely penetrating the surrounding

alveolar tissue. The migration of endothelial cells during angiogenesis, which is necessary for solid tumours to grow quickly, depends on AQP1 (Saadoun, Papadopoulos, Hara-Chikuma, et al., 2005). Due to poor angiogenesis in the AQP null animals, total tumour mass and volume in mice that spontaneously produce well-differentiated breast adenomas with lung metastases were reduced when compared to wild-type mice. Numerous additional illnesses outside of cancer have also been linked to AQP1, including hypoxia-induced angiogenesis in the retina and liver cirrhosis (Huebert et al., 2011; Ruiz-Ederra & Verkman, 2007).

The transport of glycerol or hydrogen peroxide through the AQPs channels has been linked to tumour cell proliferation (Liou & Storz, 2010; Miller et al., 2010; Papadopoulos & Saadoun, 2015; Rodrigues et al., 2016; Verkman et al., 2008), while AQPs have been suggested to directly interact with oncogenes enhancing cell division (Papadopoulos & Saadoun, 2015). Specifically, studies focused on AQP3 and AQP5 have established a correlation between these AQPs expression and tumour cell proliferation. In colorectal cancer, AQP5 is proposed to interact with the Ras pathway, which activation triggers other proteins that turn on genes involved in cell survival, differentiation, and growth (Sung et al., 2008). AQP5 has also been shown to facilitate cell proliferation and migration in lung cancer, with the involvement of EGFR/ERK/p38 MAPK signalling pathway activation (Zhang et al., 2010). After being exposed to the tumour initiator and promoter phorbol ester, cutaneous tumours in AQP3 deficient mice do not form at all. As cellular glycerol, ATP, and proliferative ability are tightly connected, glycerol supplementation restored the decreased proliferation in AQP3 deficiency (Hara-Chikuma & Verkman, 2008). In a mouse model, AQP3 knockdown inhibited tumour growth and decreased angiogenesis in human non-small cell lung cancer xenografts (Xia et al., 2014). Nevertheless, it is still unclear if the overexpression of AQPs in tumour cells is a secondary effect of higher uptake of glycerol. AQPs' water transporting function is also still a question as to whether it increases cell proliferation or not (Papadopoulos & Saadoun, 2015).

Regarding cell adhesion, a role for AQP0 is well determined, while for AQP4 it is still unclear (Papadopoulos & Saadoun, 2015). AQP0 constitutes about 50 % of the fibre cell membrane protein in lens fibre cells in the eye, and studies have revealed that the integrity and transparency of the lens depend on the interlocking protrusions' structural integrity, which is preserved by AQP0. AQP4 was proposed to have a role in cell adhesion due to structural considerations (Hiroaki et al., 2006). In an extracellular loop, AQP4 has a brief helix that enables weak connections between AQP4 molecules in neighbouring plasma cell membranes, thereby tying adjacent cells to one another. The concept that AQP4 may be involved in cell-cell adhesion is supported by the fact that expression of AQP4 causes clustering of the cells in L-cells, which lack endogenous adhesion molecules. Nevertheless, other pieces of evidence contradict these results (Zhang & Verkman, 2008). Research work demonstrates that M1-AQP4 isoforms, which are found as individual tetramers, polarize to the cell's leading edge to assist cell migration (Smith et al., 2014). On the other hand, the larger M23-AQP4 rich orthogonal arrays are bound by adhesion complexes rather than entering the lamellipodium, indicating a role for M23-AQP4 but not M1-AQP4 in cell adherence to the extracellular matrix. The conclusion is that the information on AQP4's function in cell adhesion is contradictory. Therefore, it is uncertain if AQP4 contributes to tumour cell adhesion in any way.

Taking all reported evidence together, aquaporins play a role in cancer, specifically in cellular mechanisms of migration and proliferation. Their function and ability to transport water, glycerol, hydrogen peroxide, and certain ions, induces signalling pathways, and morphologic changes, with consequences in the initiation and development of carcinogenesis. For this reason, it is believed that aquaporins may be important new therapeutic targets and/or biomarkers in prognosis in various cancer types.

1.4. Aquaporins and Signalling Pathways in Cancer

Genetic and epigenetic alterations in cellular DNA support the onset and development of cancer by causing cells to succumb to unrestrained proliferation and specific escape mechanisms that are intended for survival and migration. Human carcinogenesis causes profound changes in the signalling pathways that regulate cell motility, cell destiny, cell death, and growth and division, which ultimately catalyse the development of cancer (Moon et al., 2022). It's significant to note that during carcinogenesis, specific proto-oncogene mutations can activate oncogenes, while other types of mutations can impair the function of tumour suppressors. Numerous studies have so far shown the critical roles played by several major kinases, including the Ras-ERK and phosphoinositide 3-kinase (P13K)-Akt pathways, in the proliferation of tumour cells. Similar to this, specific signalling from the extracellular to intracellular area regulates cellular proliferation to some extent. It is now acknowledged that signalling pathways play a crucial role in other important activities, including tumour cell migration and cancer metastasis (Hüsemann et al., 2008; Moon et al., 2019; Sanchez-Laorden et al., 2014), this was initially proven by epidermal growth factor (EGF) to EGFR-mediated signalling pathways (Dittmann et al., 2005; Yarden & Sliwkowski, 2001).

The epidermal growth factor receptor (EGFR) is inserted in the ErbB family of receptor tyrosine kinases (RTKs) and is responsible for critical functions in epithelial cell physiology (Schlessinger, 2014). In different types of human cancers is usually mutated and/or overexpressed (Yarden & Pines, 2012). The studies involving EGFR determined how the intracellular signalling cascade is activated because of receptor transphosphorylation, which typically happens in response to ligand stimulation. Genes responsible for cell growth, differentiation, and survival are activated as a result of certain pathways activation that transport information from the cell surface and intracellular vesicular compartments to the nucleus (Lemmon & Schlessinger, 2010; Schlessinger, 2014; Sigismund et al., 2018).

Raf-MEK-ERK pathway is one of the pathways triggered by EGFR receptor transphosphorylation and is the best characterized among all the mitogen-activated protein kinase (MAPK) signalling pathways (Wei & Liu, 2002). This pathway is involved in molecular mechanisms such as cell cycle, proliferation, differentiation, development, and apoptosis (Guo et al., 2020; Pearson et al., 2001; Wei & Liu, 2002). When the Ras-Raf-MEK-ERK pathway is dysregulated, it promotes tumour growth, invasion, metastasis, extracellular matrix breakdown, angiogenesis, and cell survival which is linked to the oncogenesis of human cancers (Guo et al., 2020). Different forms of cancer have been linked to mutations in the Ras-Raf-MEK-ERK signalling pathway's constituent parts (Ullah et al., 2021).

The rapidly accelerated fibrosarcoma (Raf), mitogen-activated protein kinase kinase (MEK), and ERK subunits of this pathway are attractive targets for the creation of novel anti-cancer therapies due to the prominent involvement of the Raf-MEK-ERK signalling pathway in tumour genesis, maintenance, and metastasis. The therapy of various cancer forms, including melanoma, hepatocellular carcinomas, renal cell carcinoma, thyroid cancer, colorectal cancer, and glioma, uses several FDA-approved inhibitors against the elements of this pathway (Ullah et al., 2021).

Raf and MEK kinase dysregulation results in hyperactivation of ERK (Ullah et al., 2021). In addition to the upstream activation mechanisms, relatively infrequent ERK mutation or amplification was seen in ERK-inhibition-resistant cancers (Jaiswal et al., 2018; Rooney & Sethi, 2015). A significant number of cancer genetic investigations have also revealed an ERK2 D321 N mutation (Zehir et al., 2017). ERK functions as the effector kinase in the Raf-MEK-ERK cascade, which alters the cellular transcriptome and proteome by controlling a broad range of substrates (Ullah et al., 2021).

The JUN transcription factor, a substrate of the JNK MAPK pathway, and the proto-oncogene c-Fos work together to form the activator protein-1 (AP-1) heterodimer, which regulates the expression of a variety of cell cycle regulators, including cyclin D1 and p53 (Shaulian & Karin, 2001). ETS transcription factor (ELK1), a well-known ERK substrate, controls the expression of c-Fos. ELK1 is activated and

forms a complex with SRF after being phosphorylated by ERK many times. This complex in turn induces the transcription of c-Fos, thus also an ERK substrate. c-Fos is stabilized by ERK phosphorylation, which encourages neoplastic transformation (Ullah et al., 2021). The cascade is described in figure 4. (Morrison, 2012; Ullah et al., 2021) ERK pathway is associated with mechanisms involving growth, survival, differentiation, and development, while the JNK pathway is involved in inflammation, apoptosis, growth, and differentiation (Morrison, 2012).

Studies have suggested a possible association of AQPs with EGFR and ERK pathways. AQP5 was suggested to be involved in the regulation of lung cancer cells migration and angiogenesis via the EGFR/ERK1/2 signalling pathway in non-small cell lung cancer cell line H1299 (Elkhider et al., 2020).

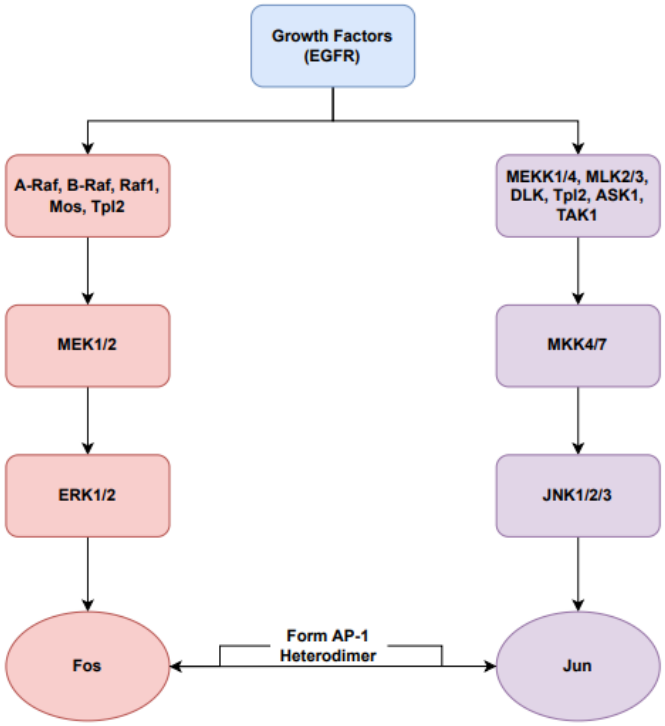

Figure 4 - MAPK Pathways. The stimulus (Growth factors) induces a cascade composed from a first level of MAPKKK (e.g. A-Raf, MEKK1/4), a second level of MAPKK (MEK1/2, MKK4/7), a third level of MAPK (ERK1/2, JNK1/2/3) and then the transcripton of genes and oncogenes (Fos, Jun), resulting in biological responses of growth, survival, differentiation, development, inflammation, and apoptosis. Adapted from Morrison (2012).

Silencing AQP5 also influenced the activation of the EGFR/ERK/p38 MAPK signalling pathway in colon and lung cancer cells (Li et al., 2018; Shi et al., 2014). In HaCaT cell line, an aneuploid immortal keratinocyte cell line from adult human skin, erlotinib, an anticancer compound, was determined to suppress the activation of the Ras/MAPK pathway and decrease the expression of AQP3, while both EGFR and ERK were also decreased in mouse skin with decreased AQP3 expression after erlotinib administration (Ikarashi et al., 2020).

EGFR phosphorylation was increased and the ERK and MAPK signalling pathways were activated in AQP5 lung cancer cells with high expression levels, while the activity of the EGFR/ERK/p38 MAPK pathway was lowered with AQP5 deletion (Zhang et al., 2010). In this study, a positive correlation was

found between the ability of cell to proliferate and AQP5 expression level. This outcome was in line with studies in which AQP5 gene silencing suppressed the EGFR/ERK/p38 MAPK signalling pathway, preventing human glioma cells from proliferating, migrating, and increasing their rate of death (Yang et al., 2017). The Ras/ERK/Rb signalling pathway may be activated in colorectal cancer to enhance the phosphorylation of the retinoblastoma (Rb) protein because of the overexpression of AQP5 (Sung et al., 2008).

In mICD-3 cells, an inner medullary collecting duct cell line from mouse transgenic, hypertonicity activated all three MAPK signalling pathways and showed that they were able to mediate AQP1 expression (Umenishi & Schrier, 2003). The inhibition of ERK, p38 kinase, or JNK pathway significantly reduced AQP1 expression, when hypertonicity was induced. Nevertheless, the study also showed that AQP1 expression, as well as AQP5 expression in another experiment in mouse lung epithelial cells, was not totally dependent on ERK pathway activation in hypertonicity conditions.

AQP8 was also detected in human oesophageal cancer Eca-109 cells, where its expression was mediated by EGFR/ERK1/2 pathway, stimulating cell invasion and migration (Chang et al., 2014).

Although there are other studies involving different signalling pathways, ERK MAPK is suggested by all this evidence to be directly involved in aquaporins expression, and in consequence, in cellular mechanisms, specifically in cellular proliferation, migration, and invasion. It is still necessary to understand the molecular mechanisms and how AQPs are able to induce the signalling pathway cascade.

1.5. Aquaporins and Epithelial-Mesenchymal Transition

During tumour development, cells go through a process called epithelial-mesenchymal transition (EMT) in which they lose epithelial characteristics and start to resemble mesenchymal cells (Pastushenko & Blanpain, 2019). Tumour start, malignant development, tumour stemness, tumour cell motility, intravasation to the circulation, metastasis, and therapeutic resistance have all been linked to EMT (Brabletz, 2012; Craene & Berx, 2013; Nieto et al., 2016). The loss of the epithelial marker E-cadherin (Ecad) and the increase in the expression of the mesenchymal marker Vimentin (Vim) are frequently used to describe EMT, which has long been thought of as a binary process involving two separate cell populations, epithelial and mesenchymal (Pastushenko & Blanpain, 2019). Recent research, however, suggests that EMT happens gradually and is characterized by a variety of cell types that express different amounts of mesenchymal and epithelial markers and have intermediate morphological, transcriptional, and epigenetic characteristics between epithelial and mesenchymal cells. Partial, incomplete, or hybrid EMT states are the states that exist between the epithelial and fully mesenchymal phases (Huang et al., 2013; Jolly et al., 2016; Pastushenko et al., 2018).

Depending on the circumstances of the pathophysiological tissue, EMT can be of three main types (Usman et al., 2021): type-1 EMT is a crucial physiological process that occurs during organogenesis and embryonic development, such as gastrulation of the exocytosis of different cell types from the neural crest; during wound healing, type-2 EMT induces cell migration, proliferation, and organ fibrosis (Rout-Pitt et al., 2018); and type-3 EMT is described in the beginning and progression of multiple diseases, including cancer and metastasis.

The expression of epithelial and mesenchymal markers in different cell lines, patient-derived xenografts, and primary malignancies has been studied (Pastushenko & Blanpain, 2019). Human primary malignancies such as breast (Livasy et al., 2006), colorectal (Kolijn et al., 2015), head and neck (Dmello et al., 2017), lung (Zacharias et al., 2018), and pancreatic cancers (George et al., 2018), as well as carcinosarcomas like uterine (Bitterman et al., 1990), kidney (DeLong et al., 1993), lung (Haraguchi et

al., 1999), breast (Bronsert et al., 2014), oesophagus (Yabuuchi et al., 2018), and skin (Paniz-Mondolfi et al., 2014) cancers have been shown to coexpress epithelial and mesenchymal markers.

Cancer cells must leave the main tumour and develop migratory and invasive skills in order to engage in the metastatic process, which is the spread of cancer cells throughout the body to plant secondary tumours at distant sites (Yilmaz & Christofori, 2009). Cancer cells use EMT, which involve a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions necessary for invasive growth, in addition to changing their adhesive repertoire, to acquire migratory and invasive properties. During EMT, immobile, polarized epithelial cells that are connected to one another by cell-cell connections separate into individual, motile, non-polarized and invasive mesenchymal cells. As a result, a cell's molecular repertoire goes though significant alterations.

Numerous extrinsic and intrinsic cues, such as gene alterations and growth factor signalling, can cause EMT (Yilmaz & Christofori, 2009). EMT can also be brought on by modifications to the extracellular matrix's chemical composition (Shintani et al., 2008). However, many aggressive, invading malignancies lack the molecular markers of EMT, suggesting that not all single cell invasions include EMT and that some tumours may only go through a partial or incomplete EMT (Tarin, 2005). Cancer cells really possess a wide variety of invasion strategies, such as amoebic or collective cell invasion, and can infiltrate even in the absence of EMT (Friedl, 2004; Wicki et al., 2006).

Snail, Twist, Zeb, and other transcription factors are up-regulated in metastatic cells that going through EMT (Boutet et al., 2007). The cell surface undergoes significant modifications as EMT-inducing gene expression rises. Ecad is a transmembrane protein that forms adherens junctions and anchors nearby cells to one another. Its cytoplasmic component is connected to the actin cytoskeleton by α- and β-catenin (Heerboth et al., 2015). EMT requires the loss of Ecad, which is also a crucial component of metastatic cells (Onder et al., 2008). Individual cells are free to migrate when there are no tight adherens junctions holding tissues together, which is essential for cancer spread. During this phase, there is also a rise in the expression of N-cadherin and Vim, two proteins thought to be indicators of mesenchymal phenotype and essential for cellular migration (Heerboth et al., 2015).

Along with other cytoskeletal elements including microfilaments and microtubules, vimentin is a crucial type III intermediate filament protein (Usman et al., 2021). It is widely acknowledged that it plays a dynamic role in a variety of key cellular mechanisms, including structural support, attachment, migration, and signalling (Danielsson et al., 2018). Vimentin is widely regarded as a classic biomarker of type-3 EMT since it is regularly seen to be overexpressed during cancer metastasis (Liu et al., 2015; Wu et al., 2018). Numerous studies have emphasized its crucial part in the control of this intricate process (Vuoriluoto et al., 2011). By creating a viscoelastic framework, vimentin filaments shield cancer cells from mechanical stresses experienced during migration or squeezing through small spaces (Patteson et al., 2019). They also support the location and integrity of organelles, particularly the nucleus, during EMT and cancer growth (Pattabiraman et al., 2020).

A number of secreted substances play crucial roles in both the maintenance of EMT and the encouragement of metastasis (Chaffer & Weinberg, 2011). Matrix metalloproteinases (MMPs) have the ability to cleave cell-surface proteins and degrade extracellular matrix elements, enabling migratory cells to penetrate surrounding tissues and breach the basement membrane (Brinckerhoff & Matrisian, 2002). Ecad is a crucial MMP substrate because its cleavage promotes signalling that supports EMT in addition to assisting in the division of tissues into individual cells. It has been demonstrated that cleavage of the Ecad ectodomain produces a fragment called sEcad that can activate EGFR signalling in a paracrine manner to promote EMT, invasion, and proliferation (David & Rajasekaran, 2012; Noë et al., 2001).

Aquaporins have also been studied alongside E-cad and Vim, to determine a possible association with EMT. In pancreatic cancer cells (Direito et al., 2017), it was possible to observe a AQP3 and AQP5 overexpression accompanied by a downregulation of E-cad and an upregulation of Vim. Poorly

differentiated tumours showed lower expression levels of E-cad compared to moderately differentiated ones, with opposite change in Vim expression, indicating the occurrence of EMT. The results suggest that AQP5 and AQP3 participates in the process of EMT in pancreatic cancer. Regarding AQP5, this was already observed in BEAS-2B human bronchial epithelial cells expressing AQP5 that exhibited loss of E-cad and gain of Vim, accompanied by changes in the morphology, which indicated an AQP5 stimulus in bronchial epithelial cells to undergo EMT (Chae et al., 2008).

In gastric cancer (Chen et al., 2014), AQP3 was also overexpressed in tumour tissues compared with normal gastric mucosa, while E-cad was down-regulated. Although Vim was only detected in 14 out of 89 cases studied, it was only expressed in carcinoma tissues that overexpressed AQP3 and lacked expression of E-cad and was not detected in normal gastric glands. Moreover, AQP3 expression positively correlated with Vim expression in gastric cancer tissues, while it was inversely correlated with E-cad expression. This evidence together indicate a possible involvement of AQP3 in induction of EMT in gastric cancer.

A study on colorectal cancer (CRC) (Chen et al., 2017), it was possible to observe in human colon cancer cell lines SW480 and HCT-116 the overexpression of AQP5 promoting the mesenchymal-like phenotype and EMT process, and the silence of AQP5 resulted in the inhibition of EMT in the same cell lines. Furthermore, Cairicoside E (CE), a resin glycoside isolated from Ipomoea cairica, and inhibitor for proliferation in multiple tumour cells, was able to downregulate AQP5 expression and suppress EMT in CRC cells. The inhibitory effect of CE on EMT was reversed by AQ5 overexpression, however, when AQP5 was silenced, CE had no impact on EMT. This evidence show that the inhibitory effect on EMT depends on the AQP5 expression downregulation. CE was also able to inhibit AQP5 and Vim expression in lung metastatic nodules, confirming its possible inhibitory role on metastasis through AQP5 downregulation in vivo.

In multiple types of cancer, AQPs are suggested to be influential on EMT, not only inducting the process but also inhibiting it when downregulated. The mechanisms involving this association are still unclear, but AQPs may have a role as new therapeutic targets in metastasis or biomarkers to better understand the invasive or aggressive grade of the tumour, through the classification of EMT type in the tumour.

1.6. Aquaporins in Pancreatic Cancer

The pancreas is a key organ for overall body homeostasis, with both endocrine and exocrine functions (Arsenijevic et al., 2019). Endocrine pancreatic β-islets contribute to glucose homeostasis, secreting insulin in response to hyperglycaemia (Henquin, 2011), whereas exocrine pancreatic acinar and ductal cells secret pancreatic fluid composed of water, enzymes essential to food digestion, and ions (Hegyi & Petersen, 2013).

Aquaporins are expressed in the endo- and exocrine pancreas, ensuring the correct physiological function of insulin and pancreatic fluid secretion (Bruun-Sørensen et al., 2021). While AQPs distribution remains unknown in the human endocrine pancreas, it has been identified in mouse and rat β-cells (AQP5, AQP7, AQP8, and AQP12). Regarding the exocrine pancreas, AQPs mRNA was found in human tissues (AQP1, AQP3, AQP4, AQP5, AQP8, and AQP12). However, only 3 aquaporins proteins were detected (Burghardt, 2003; Direito et al., 2017). AQP1 was identified in centroacinar cells, intercalated ductal cells, capillary endothelial cells, and pancreatic zymogen granule membranes. AQP5 was detected in intercalated ductal cells, while AQP8 was localized only in acinar cells. More recently, AQP3 was also detected in a portion of acinar cells. The distribution of aquaporins in a healthy human pancreas is illustrated in figure 5.

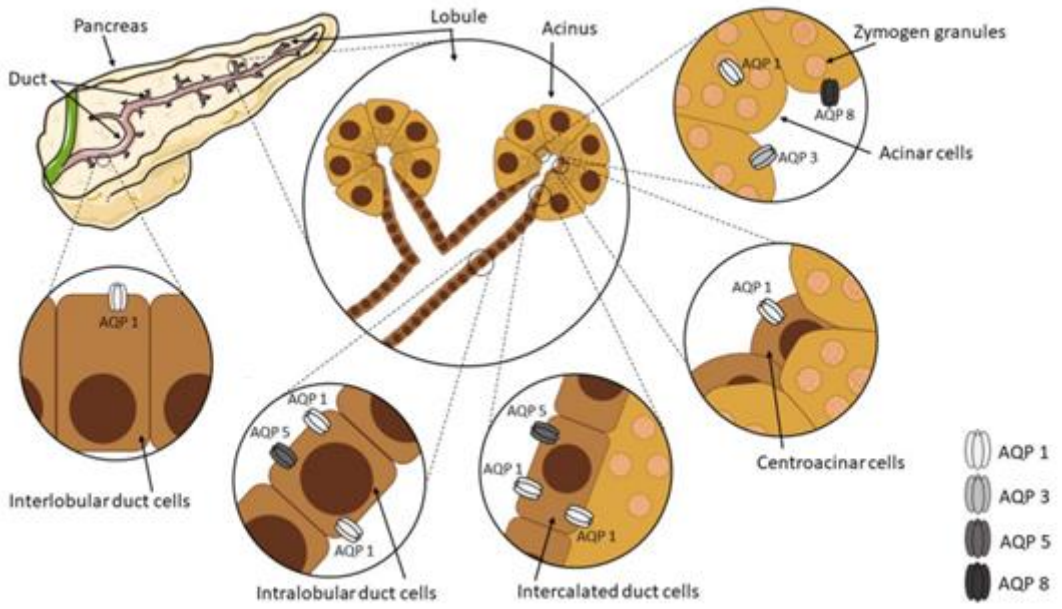

Figure 5 - AQP expression in a healthy human exocrine pancreas is shown schematically. The pancreas is depicted in cross-section with lobules and intralocular ducts, as well as a magnified view of one of the lobules. The apical and basolateral membranes of interlobular duct cells and intercalated duct cells expresses AQP1, while AQP5 is expressed at the apical membrane. AQP1 is expressed in the apical membrane of centroacinar cells. The apical membrane of acinar cells expresses AQP8. Recently, AQP3 was discovered to be expressed in the membrane of a portion of acinar cells in adittion to AQP1 being expressed in the mebrane of zymogen granules. Adapted from Bruun-Sørensen (2021).

The exocrine pancreas, which makes up around 90 % of all pancreatic cells, is made up of acinar and ductal epithelial cells that secrete pancreatic juice, which contains fluid and enzymes and amounts to about 1 to 2 litres daily. For effective food digestions and the neutralization of stomach acid, pancreatic

juice secretion is necessary. Several neurotransmitters, including secretin, cholecystokinin, and acetylcholine, regulate the secretion of pancreatic juice (Arsenijevic et al., 2019; Konturek et al., 2003).

Acinar cells secrete an isotonic fluid as the first step in the process of secreting pancreatic juice, while ductal cells secrete the majority of the water along with sodium, chloride, and bicarbonate ions as the second step. Acinar AQP8 (located apically), ductal AQP1 (apically and basolaterally placed), and AQP5 (located apically) all work together to enable transcellular water flow to the gland lumen in this two-step procedure (Burghardt et al., 2006; Lee et al., 2012).

A number of pancreatic conditions, including cancer, cystic fibrosis, and pancreatitis, have been linked to AQPs (Arsenijevic et al., 2019). With a five-year survival rate of only 2-9 %, pancreatic cancer is the seventh most lethal type of cancer and the 14th most prevalent overall (Ilic & Ilic, 2016). A significant factor in the low survival rate is the late diagnosis brought on by vague symptoms, which in 80-85 % of instances results in unresectable tumours. 90 % of pancreatic tumours are pancreatic ductal adenocarcinomas (PDAC) (McGuigan et al., 2018).

PDAC develops when cells differentiate and invade the parenchyma in the pancreatic ductal tree. 60-70 % of all instances of PDAC begin in the head of the pancreas, and these cases are frequently accompanied by invasion of the gall and Wirsung ducts (Luchini et al., 2016). More advanced cases of PDAC also spread to the papilla of Vater, the wall of the duodenum, the retroperitoneal adipose tissue, and the lymph nodes. 15 % of the remaining cases begin in the pancreas' body, while another 15 % do so in the organ's tail. These PDACs frequently have larger tumour masses that clog the Wirsung duct and expand to the lymph nodes, spleen, stomach, left adrenal gland, peritoneum, colon, and other organs.

Pancreatic cancer is predicted to become the second greatest cause of cancer-related death by 2030 (Siegel et al., 2018). The only therapeutic option for treating PDAC at this time is surgery and due to the lack of distinguishing symptoms and clear clinical signs for early stages of PDAC, early PDAC diagnosis is still challenging (Arsenijevic et al., 2019).

Following the reports on PDAC cell lines, it was hypothesized that AQPs were involved in the migration, proliferation, and enhanced apoptosis of PDAC cells (Burghardt, 2003; Huang et al., 2017; Liu et al., 2012). In contrast to normal pancreatic tissues, intercalated and intralobular ductal cells revealed altered AQP5 location and expression in preliminary research employing PDAC from a small cohort of Caucasian patients (Direito et al., 2017). In fact, AQP5 labelling was distributed intracellularly and throughout the entire plasma membrane, as opposed to the typical apical membrane in healthy pancreatic tissue. Additionally, PDAC had greater levels of AQP5 expression, which was associated with the tumour differentiation status and aggressiveness. When compared to normal pancreatic tissues, the same PDAC also showed altered AQP3 location and expression. Indeed, in PDAC (although diverse among PDAC samples), AQP3 labelling was confined to ductal cells (plasma membrane or intracellularly), whereas in normal pancreatic ductal cells, it was almost non-existent. Additionally, tumour differentiation status and aggressiveness were negatively linked with AQP3 expression.

When compared to benign pancreatic tissue, PDAC from a second study's relatively larger cohort of Chinese patients (Zou et al., 2019) showed higher AQP1 and AQP3 expression, and the expression level was inversely correlated with the disease's tumour and node metastasis stages. Positive AQP1 and AQP3 expression were also substantially linked to patient survival, but they also had a poor prognosis in PDAC.

Therefore, PDAC development and progression may be impacted by AQP over- and ectopic expression, just like in a number of other malignancies. Aside from AQP1, AQP3, and AQP5, PDAC tissues have also shown lower levels of AQP7 than normal pancreatic tissue, according to data from 142 primary

tumour samples and 104 samples of normal pancreatic tissue obtained using transcriptome microarray (Magouliotis et al., 2019).

The usefulness of employing AQPs as markers of pancreatic cancer stages and prognosis needs to be further evaluated using bigger cohorts of pancreatic cancer patients. More research will be needed to understand the molecular mechanisms driving the differential expression of AQPs in pancreatic cancer and the advantages of targeting AQPs in pancreatic cancer with the right treatment tools (Arsenijevic et al., 2019).

1.7. Aquaporins as Biomarkers

Aquaporins are expressed in many tissues and are overexpressed in various cancers, and is associated with overall survival, poor survival, pathological grade, clinical stage and even resistance to chemotherapy (Arsenijevic et al., 2019). Therefore, the research on aquaporins in those cancers have obtain evidence on the value of AQPs as biomarkers of diagnosis and prognosis. Cancer diagnosis and treatment monitoring benefit greatly from the use of prognostic and predictive biomarkers.

AQP3, AQP5, AQP6, AQP8, AQP10, and AQP11 mRNA expression were significantly correlated to favourable overall survival (OS) in ovarian cancer patients (Chetry et al., 2018), whereas AQP0, AQP1, and AQP4 mRNA were associated with poor survival analysis platform of Kaplan-Meier plotter. Additionally, it has been found that ovarian cancer patients receiving concurrent-based chemotherapy, Platin, Taxol, and high levels of AQPs had important prognostic impacts. Additionally, it was observed that certain AQPs had a crucial role in predicting outcomes for patients with ovarian cancer in both early and advances clinical stages and at various pathological grades. These findings showed that every AQP, with exception of AQP2 and AQP9, was associated with a distinct prognostic relevance and may therefore serve as new prognostic indicators for ovarian cancer.

Another study revealed a substantial correlation between the protein and mRNA expression of the AQPs and the prognosis of patients with gastric cancer (Thapa et al., 2018). A thorough analysis of each individual AQP in the Protein Atlas database revealed that higher mRNA expression of AQP3, AQP9, and AQP11 was associated with better OS in all gastric patients, whereas higher mRNA expression of AQP0, AQP1, AQP4, AQP5, AQP6, AQP8, and AQP10 was associated with worse OS. Additionally, AQPs demonstrated key predictive events in patients with gastric cancer who had a variety of clinicopathological characteristics, including Laurens classification, gender, pathological grade, clinical stage, human epidermal growth factor receptor 2 (HER2) status, and therapy preferences. A membrane tyrosine kinase and oncogene, HER2 contributes to aggressiveness in tumours by activating powerful proliferative and anti-apoptotic signals to cells (Dajani et al., 2018).

Furthermore, it was possible to distinct between hepatocellular carcinoma, cholangiocarcinoma, and metastatic colorectal carcinomas in hepatic malignancy by immunohistochemistry detection of AQP1 (Wang et al., 2015). Whereas reduced AQP9 expression in hepatocellular carcinoma may be a sign of resistance to apoptotic stimulation. Patients with hepatocellular carcinoma who overexpress AQP3 and AQP5 have poor prognosis and low 5-year survival rates (Dajani et al., 2018).

AQP1 levels in urine can also be used as biomarker for renal cell cancer, since urine AQP1 concentration is 12 times higher in people who have known renal cell cancer (Morrissey et al., 2015). Patients with cutaneous melanoma who tested positive for AQP1 have been reported to have a lower progression-free and overall survival rate. In cutaneous melanoma, AQP1 expression has also been linked to poor prognosis (Imrédi et al., 2016).

In colorectal cancer and lung adenocarcinoma (Tan et al., 2014), AQP5 is overexpressed. It showed high levels of expression in 14 samples, moderate levels of expression in 29 samples, and no expression

in 2 samples in a cohort of 45 colorectal cancer tumours. Increased expression levels were related to lymph node metastasis, distant metastasis, and TNM stage, which is the categorization of malignant tumours, suggesting that AQP5 expression level may function as prognostic marker. Additionally, the cumulative survival rate was higher for patients who did not express AQP5. Furthermore, AQP5 might be used as prognostic indicator for ductal breast cancer. In other hand, when early breast cancer with HER2 overexpression undergoes curative surgery, AQP3 has shown promise as a potential prognostic marker (Kang et al., 2015).

More than 90 % of pancreatic cancer patients are diagnosed in stages III and IV, which contributes to pancreatic cancer's dismal prognosis (Kikuyama et al., 2018). Prognosis is valuable because it determines the outcome of the disease, optimizes the treatment strategies, and gives the patient the information needed for management and decision-making (Halabi & Owzar, 2010). For this reason, early diagnosis is necessary to increase prognosis, and it is important to understand the clinical signs and symptoms that point to pancreatic cancer in order to make an early diagnosis and identify people who are most at risk for the disease (Kikuyama et al., 2018).

A study evaluated the expression of AQPs on 106 samples of PDAC tissue that had been resected and found that PDAC had higher levels of AQP1 and AQP3 expression than peritumoral tissue or healthy pancreatic tissue. Both AQP1 and AQP3 are suggested as diagnostic indicators of PDAC as well as indicators of patients with PDAC poor prognosis (Zou et al., 2019). A second study that involved 35 PDAC patients found that AQP3 was associated with the later, more aggressive phases of the disease, whereas AQP5 was suggested as a potential histology marker for the earlier stages of the disease (Direito et al., 2017).

According to a different study, miR-874 inhibits AQP3 expression in PDAC, which causes PDAC cell lines to proliferate less and undergo more cell death. The predictive value of AQP3 should be further evaluated in larger cohort of clinical samples (Huang et al., 2017).

Finally, it is hypothesized that AQPs modulation may play a role in some cancer cell's capacity to respond to therapy (Arsenijevic et al., 2019). For instance, it has been demonstrated that AQP3 overexpression contributes to chemo-resistance to arsenite in melanoma (Gao et al., 2012) and to cisplatin in gastric cancer (Dong et al., 2016). By blocking the Wnt-β-catenin signalling pathway, AQP5 knockdown makes colorectal cancer cells more susceptible to 5-fluorouracil (Li et al., 2018).

It's not clear the influence of aquaporins in resistance to treatment, nor is validated the use of aquaporins for biomarkers in cancer. Further investigation will provide resolution to how to better understand aquaporins involvement in cancer, relying on these proteins as biomarkers, specially of prognosis.

2. Thesis Aim

The global aim of this research work is the validation of aquaporins as possible prognosis and/or diagnosis biomarkers in pancreatic cancer.

The first objective is to determine aquaporins' expression in pancreatic cancer. The expression will be assessed in 25-paired healthy and tumour tissues obtained by surgical resection during pancreatic cancer surgery. AQP1, AQP3, and AQP5 are known to be expressed in the pancreas (both healthy and malignant) and with evidence of being involved in pancreatic cancer. AQP9 expression is still unknown in the pancreas and pancreatic cancer.

The second objective is to determine aquaporins' expression interplay with the pathways involved in tumorigenesis. Due to the association with AQP1, AQP3, and AQP5 with EGFR and ERK MAPK pathway in tumorigenesis in various cancer types, EGFR, ERK1, ERK2, c-Jun and c-Fos expression will be evaluated. Ecad and Vim are the EMT biomarkers chosen to evaluate the association of AQPs with cancer progression.

Healthy and tumour tissues will be homogenized in TRIzol and messenger RNA (mRNA) will be extracted using the chemical extraction protocol (chloroform-isopropanol). After mRNA concentration and quality are evaluated, reverse transcriptase will be performed under NZYTech protocol. All proteins' relative expression will be assessed through Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). The collected data will be analysed for significant multiple comparisons with robust statistical tools.

Understanding and studying the relative expression of each aquaporin, signalling pathway agent and EMT biomarker in healthy and tumour tissues will complete the second objective. Comparing the relative expression of each aquaporin in healthy and tumour tissues will complete the third and last task, of validating aquaporins as possible biomarkers in pancreatic cancer.

3. Methods

3.1. Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

3.2. Cohort

In a collaboration with a team of surgeons, tumour samples and matched adjacent non-neoplastic samples were obtained from 25 patients who underwent curative surgery for multiple types of pancreatic cancer, between November 2012 and March 2013. The patients' features are described in table 2. All patients were classified according to the aggressiveness and invasiveness grades of cancer. The samples drove from 13 male patients and 12 female patients, with an age range from 51 to 82 years old at the time of the surgery. The most common cancer type is Ductal Adenocarcinoma, with a total of 9 patients suffering from this disease out of the total 25.

Table 2 - Description of Patient's features, cancer type, aggressiveness grade and invasiveness grade.

Patient	Sex	Age	Cancer Type	Aggressiveness Grade[1]	Invasiveness Grade[2]
1	M	69	Ampulla Adenocarcinoma	Low	High
2	M	55	Kidney Metastasis	Low	Low
3	F	55	Ductal Adenocarcinoma	High	High
4	M	63	Ampulla Adenocarcinoma	Low	High
5	M	69	Distal Cholangiocarcinoma	High	High
6	M	59	Ductal Adenocarcinoma	High	High
7	M	69	-	-	-
8	F	69	Non-invasive Intraductal Papillary Mucinous Neoplasm	Low	Low
9	F	52	Invasive Intraductal Papillary Mucinous Neoplasm	Low	High
10	F	69	Endocrine Neoplasia	High	Low

[1] The aggressiveness grade was defined depending on the subjective evaluation from the performer surgeon, that classified that specific tumour on a scale from 1 to 4 aggressiveness. The Low-aggressive grade group constitutes all patients with cancer with an aggressive grade ≤ 2; the High-aggressive grade group constitutes all patients with cancer with an aggressive grade ≥ 3.

[2] The invasiveness grade was defined depending on the histological grade of each tumour. The Low-invasive grade group constitutes all patients with cancer with a histological grade = 1; the High-invasive grade group constitutes all patients with cancer with a histological grade ≥ 2.

11	F	82	Ductal Adenocarcinoma	High	High
12	M	58	Distal Cholangiocarcinoma	Low	High
13	F	65	Non-invasive Intraductal Papillary Mucinous Neoplasm	Low	Low
14	F	65	Ductal Adenocarcinoma	High	High
15	M	80	Ductal Adenocarcinoma	Low	High
16	F	72	Ampulla Adenocarcinoma	Low	High
17	F	60	Ductal Adenocarcinoma	High	High
18	M	56	Ampulla Adenocarcinoma	Low	High
19	M	56	Cystic Neuroendocrine Tumour	Low	Low
20	M	67	Ductal Adenocarcinoma	High	High
21	F	78	Ductal Adenocarcinoma	High	High
22	M	50	Distal Cholangiocarcinoma	Low	High
23	F	80	Invasive Intraductal Papillary Mucinous Neoplasm	Low	Low
24	M	64	Ductal Adenocarcinoma	High	High
25	F	51	Cystic Neuroendocrine Tumour	Low	Low

3.3. RNA Extraction

Healthy and tumour tissue samples were stored at -20º C in 500 µL TRIzol (NZYOL, NZYTech, Lisbon, Portugal). After defrosting in ice, the samples were homogenized with TissueRuptor (Qiagen, Hilden, Germany).

To each sample was added 100 µL chloroform, homogenized for 15 seconds, rested for 2 minutes at room temperature, and centrifuged (Eppendorf Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 15 minutes, at 4 ºC and 10,000 rpm. Two phases were formed, the aqueous phase (upper layer) was pipetted to a new 2 mL sterile nuclease-free microtube, to which was slowly added 250 µL isopropanol. After quick homogenization, the samples were centrifuged for 10 minutes, at 4 ºC and 10,000 rpm. The supernatant was discarded, 500 µL ethanol 75 % was added and the samples were centrifuged for 5 minutes, at 4 ºC and 10,000 rpm. The supernatant was discarded and both steps were repeated. After discarding the supernatant, the microtubes were left to air dry for at least 30 minutes, following a 10-minute period in a dry bath (Accublock, Labnet, Edison, USA) at 55º C, and the addition of 10 µL diethylpyrocarbonate-treated water (DEPC-treated water) (NZYTech, Lisbon, Portugal).

3.4. Reverse Transcriptase – Complementary DNA Synthesis

Complementary DNA (cDNA) is a copy of DNA originated by the enzyme Reverse Transcriptase, using messenger RNA (mRNA) or single-stranded RNA (ssRNA) as the template. These molecules are useful for studying specific genes expression since it contains only certain fragments of the genome.

RNA concentrations were quantified in a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Waltham, USA) and the reverse transcription was performed with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). The mixtures were prepared with 500 ng RNA with quality ratios 260/280 and 260/230 between 1.8-2.2. The RNA template was added accordingly to 10 µL NZYRT Reaction Mix, 2 µL NZYRT Enzyme Mix and DEPC-treated water, if necessary, completing 20 µL, in a 200 µL sterile, nuclease-free PCR microtube (GRiSP GRS 0.2 mL PCR Tubes, Oporto, Portugal). The reaction was performed in a CFX96 Touch Real-Time PCR Detection System (BioRad, California, USA), following the conditions described in table 3.

Table 3 - Reverse Transcriptase Reaction's Protocol Part 1 Conditions.

Temperature (ºC)	Duration (Minutes)
25	10
50	30
85	5
4	∞

After the run was complete, it was added to each microtube 1 µL NZYRT RNAse H (*E. coli*). The microtubes were placed again in the Thermal Cycler, following the conditions described in table 4.

Table 4 - Reverse Transcriptase Reaction's Protocol Part 2 Conditions.

Temperature (ºC)	Duration (Minutes)
37	20
4	∞

The resulting cDNA samples were stored at 4 ºC.

3.5. Real-Time Quantitative Polymerase Chain Reaction (RT qPCR)

Transcript levels were quantified by real-time quantitative PCR in a QuantStudio 7 Flex (Applied Biosystems, Waltham, USA).

The total PCR reaction volume for each cDNA template was 6 µL and was applied in a 384 well plate (Thermo Scientific Sigma-Aldrich, Darmstadt, Germany) with a repetitive pipette BRAND HandyStep S (Sigma-Aldrich, Darmstadt, Germany).

AQP1, AQP3, AQP5, AQP9 and β-actin (Housekeeping Gene) were quantified using TaqMan RT qPCR assay. The PCR reaction volume was prepared with 2.7 µL of TaqMan Universal Master Mix with UNG (Thermo Fisher Scientific, Waltham, USA), 0.3 µL of TaqMan Gene Expression Assay, described for each gene in table 5, and 3 µL of cDNA template diluted 1:8 in DEPC-treated water.

Table 5 - Aquaporins' and corresponding housekeeing gene's specific probes used for RT-qPCR.

Gene	Full Gene Name	TaqMan Gene Expression Assay
AQP1	Aquaporin 1	Hs01028916_m1
AQP3	Aquaporin 3	Hs01105469_g1
AQP5	Aquaporin 5	Hs00387048_m1
AQP9	Aquaporin 9	Hs00175573_m1
Housekeeping Gene		
ACTB	β-actin	Hs99999903_m1

EGFR, ERK1, ERK2, c-Fos, c-Jun, E-cad, Vim and HPRT1 (Housekeeping gene) were quantified using Sybr Green RT qPCR method. The PCR reaction volume was prepared with 3 µL of SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 1 µL of the mixture of Forward/Reverse Primers, described in table 6 (Lemos, 2018), and 2 µL of cDNA template diluted 1:8 in DEPC-treated water.

Table 6 - Genes' and corresponding housekeeping gene's specific primers used for RT-qPCR.

Gene	Full Gene Name	Forward/Reverse Primers
EGFR	Epidermal growth factor receptor	F: 5' GAAATCCTCGATGAAGCCTACGTG '3
		R: 5' GTCTTTGTGTTCCCGGACATAGTC '3
ERK1	Extracellular signal-regulated kinase 1	F: 5' AAGATCAGCCCCTTCGAACATC '3
		R: 5' CTTGTACAGGTCAGTCTCCATCAG '3
ERK2	Extracellular signal-regulated kinase 2	F: 5' TACACCAACCTCTCGTAACATC '3
		R: 5' CATGTCTGAAGCGCAGTAAGATT '3
E-cad	E-cadherin	F: 5' TCGACACCCGATTCAAAGTG '3
		R: 5' GTCCCAGGCGTAGACCAAGA '3
Vim	Vimentin	F: 5' TGCCCTTAAAGGAACCAATGAG '3
		R: 5' AGGCGGCCAATAGTGTCTTG '3
c-Jun	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	F: 5' GTATCCTGCCCAGTGTTGTTTG '3
		R: 5' GCAGAAAAGAGGTTAGGGGAGTAC 3'
c-Fos	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	F: 5' CCGGGGATAGCCTCTCTTACT '3
		R: 5' CCAGGTCCGTGCAGAAGTC '3
House-keeping Gene		
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase 1	F: 5'ACTGAACGTCTTGCTCGAGATG '3
		R: 5' AGCAGGTCAGCAAAGAATTTATAGC '3

All samples were run in duplicates. The amplification was performed in the conditions described in table 7. After step 4 was completed, step 3 followed and so forward until the 45 cycles were finished.

Table 7 - Real-Time Quantitative Polymerase Chain Reaction's Protocol Conditions.

Step	Temperature (ºC)	Duration	Cycles
(1)	50	2 minutes	1
(2) Initial Denaturation	95	3 minutes	1
(3) Denaturation	95	10 seconds	45
(4) Annealing/Extension	60	1 minute	45
(5)	4	∞	

3.6. CT Analysis – Lyvak Method

In RT qPCR, the program will determine the point in time when the amplification of the gene target is first detected – the Threshold Cycle (CT). This parameter is defined as the fractional cycle number at which the fluorescence, produced by the gene amplification, passes the fixed threshold. This threshold is established at the initial PCR cycles when the change in fluorescence signal is little and the baseline for the amplification plot is defined. When the reaction is finished, each duplicate will have a determined CT value.

The mean genes' relative expression was calculated using a variation of the Livak Method (Livak & Schmittgen, 2001), corrected for variation in amplification efficiency, as described by Fleige and Pfaffl (Fleige & Pfaffl, 2006):

$$Relative\ expression\ = \frac{E^{CT}(Housekeeping\ Gene)}{E^{CT}(Target\ Gene)}\ ,where\ E = 1 + Efficiency\ of\ Reaction$$

3.7. Statistical Analysis

Statistics was carried out with the Generalised Linear Mixed (GLM) model of Statistical Analysis System (SAS) software, version 9.4 (SAS Institute, Cary, NC, USA). Once normality was tested by Kolmogorov-Smirnov test and variance homogeneity by Levene's Test, significant multiple comparisons test was carried out using the PDIFF option adjusted with Tukey-Kramer to determine statistical differences among the healthy and tumour tissues. Pearson's correlation coefficients were calculated with the Proc CORR procedure to establish linear relationships among gene expression. All data were presented as mean and standard error of the mean (SEM). A *P* value lower than 0.05 was considered statistically significant.

4. Results

4.1. Tumour vs. Healthy Tissues

4.1.1. Aquaporins

All aquaporins were found to be expressed in both healthy and tumour tissues.

AQP1 suffered a relative expression level decrease, from a value of 1.05 in healthy tissues (HT) to 0.261 in tumour tissues (TT), of 75.1 %. This decrease was statistically significant (P = 0.003).

AQP3 presents similar values in both tissue types (HT = 0.271; TT = 0.223), with a slightly decrease in tumour tissue of 17.7 %. This decrease was not statistically significant (P = 0.787).

AQP5 is overexpressed in tumour tissues (HT = 0.208; TT = 0.627), increasing 201 %. However, the results were not statistically significant (P = 0.354).

This research confirms that AQP9 mRNA is found in these tissues. AQP9 relative expression increases in tumour tissues in 53.7 % (HT = 0.134; TT = 0.206). Although the results are not statistically significant (P = 0.759), these results indicate the possibility of AQP9 also being involved in the carcinogenesis of pancreatic cancer. All results are represented in figure 6.

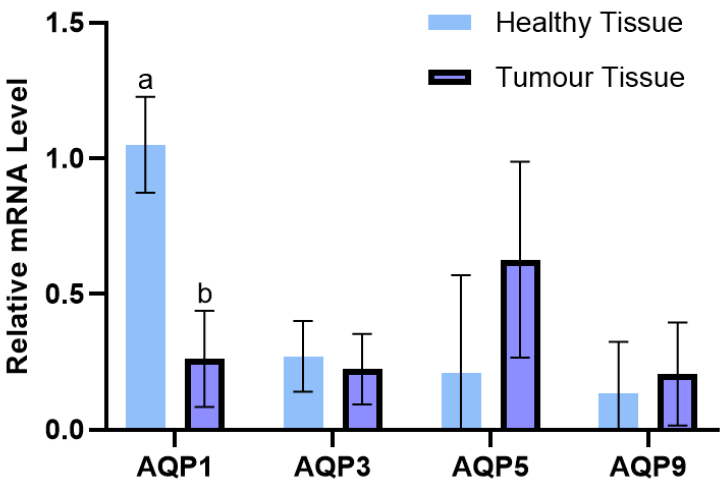

Figure 6 - Effect of tissue on the relative gene expression of AQP1, AQP3, AQP5, and AQP9. Healthy tissues are represented in light blue without a border; tumour tissues are represented in purple with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.1.2. E-cadherin and Vimentin

E-cadherin and vimentin are both expressed in healthy and tumour tissues. All results are represented in figure 7.

Ecad relative expression increases in 124 % (HT = 0.827; TT = 1.85). However, the results are not statistically significant (P = 0.108).

Vim is also expressed in both tissue types (HT = 10.5; TT = 17.9), increasing 70.5 % in tumour tissues. However, the results are not significantly different (P = 0.240).

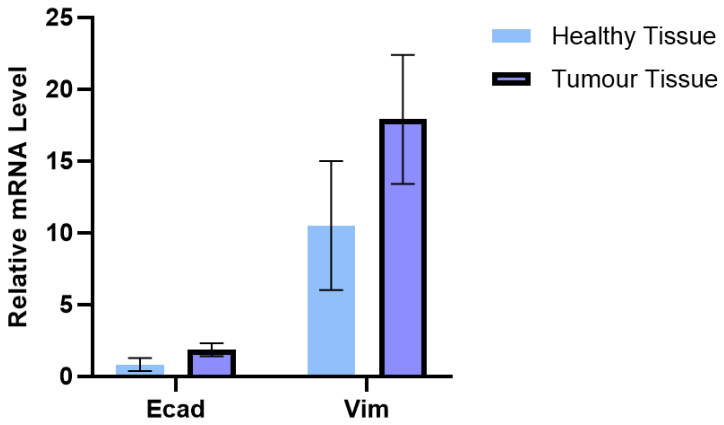

Figure 7 - Effect of tissue on the relative gene expression of E-cadherin and Vimentin. Healthy tissues are represented in light blue without a border; tumour tissues are represented in purple with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.1.3. EGFR and ERK MAPK Pathway Proteins

EGFR, ERK1, ERK2, c-Jun and c-Fos mRNA were detected in healthy and tumour tissues. All results are represented in figure 8.

EGFR is overexpressed in tumour tissues (HT = 2.25; TT = 3.95), corroborating the previous studies. The same behaviour was detected for ERK2 (HT = 2.82; TT = 3.9). Both ERK1 and c-Jun relative expression decreased in tumour tissues, with a decrease of 7,84 % (HT = 10.2; TT = 9.4) and 15.6 % (HT = 4.73; TT = 3.99) respectively. c-Fos presents similar values for both tissue types, with a slightly increase in tumour tissues of 4.15 % (HT = 0.627; TT = 0.653). All genes were expected to be overexpressed in tumour tissues, and the results for each gene are not statistically significant (P > 0.05).

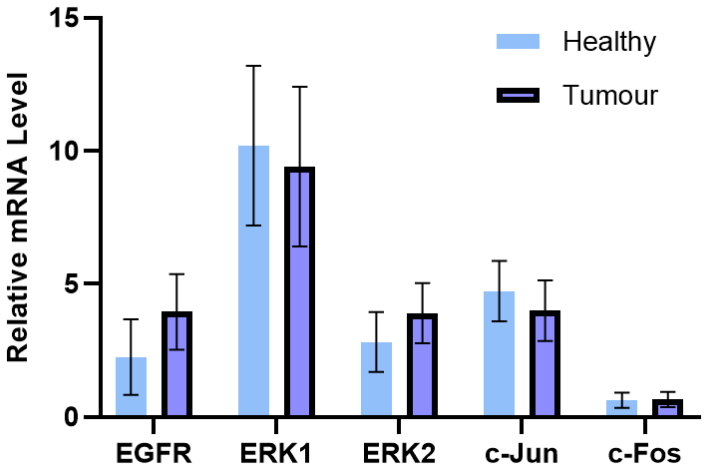

Figure 8 - Effect of tissue on the relative gene expression of EGFR, ERK1, ERK2, c-Jun, and c-Fos. Healthy tissues are represented in light blue without a border; tumour tissues are represented in purple with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.2. Age and Gender in Tumour and Healthy Tissues

4.2.1. Aquaporins

Aquaporins' relative expression is not significantly different when evaluating age. The patients were divided into two groups of age, guaranteeing two groups of similar number: patients younger than or with 65 years old (14 patients – Young Group), and patients older than 65 years old (11 patients – Old Group). All results are represented in figure 9. In both tissue types, the results were not statistically different. However, it is important to note the increase of AQP5's relative expression in tumour tissue of 278 % (Young Group = 0.270; Old Group = 1.02), especially because AQP5 relative expression level in Old Group is 0.010 (increase of 374 % in tumour tissues), while in Young Group is 0.297 (decreasing 9,09 % in tumour tissues).

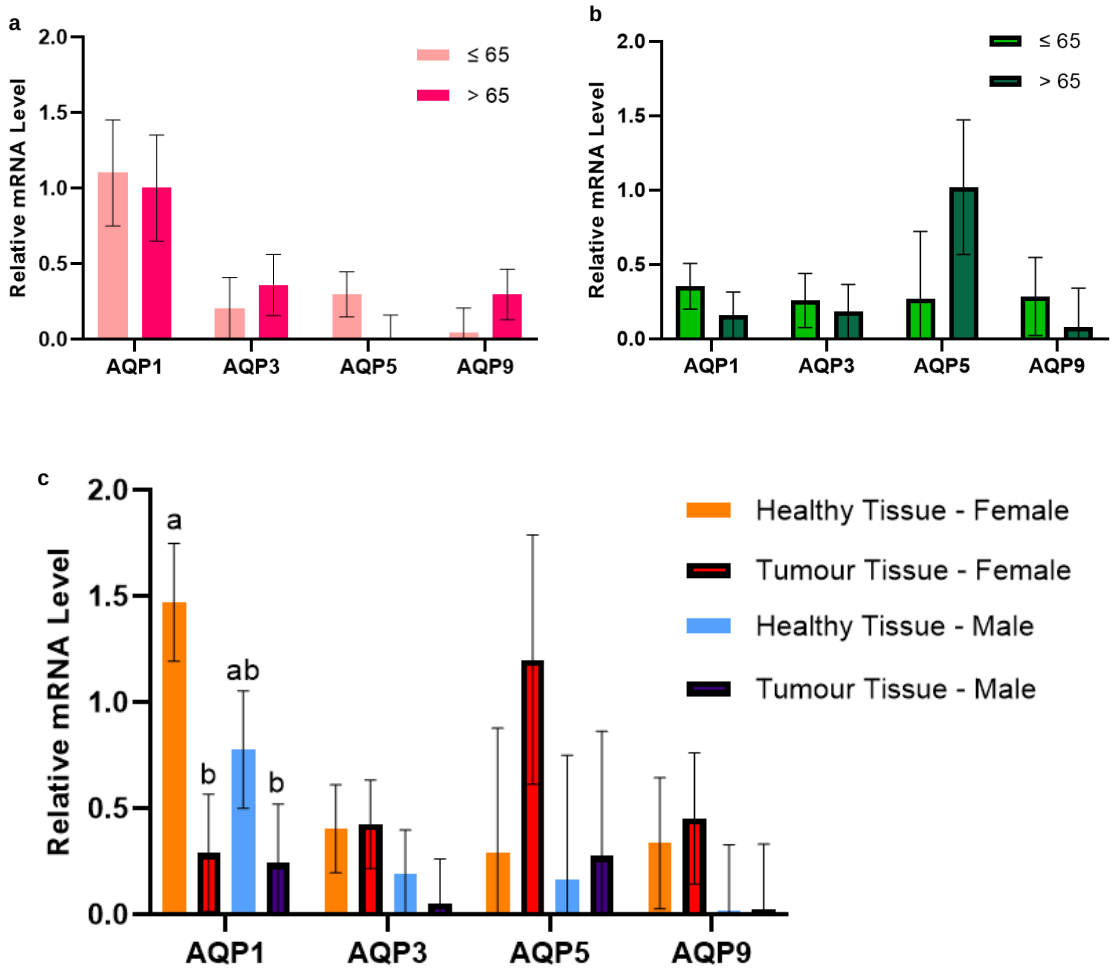

Figure 9 - Effect of tissue, age, and gender on the relative gene expression of AQP1, AQP3, AQP5, and AQP9. a Effect of age in healthy tissues. The Young Group (≤ 65 years old) are represented in light rose without a border; the Old Group (> 65 years old) is represented in dark rose without a border. b Effect of age in tumour tissues. The Young Group (≤ 65 years old) are represented in light green with a black border; the Old Group (> 65 years old) is represented in dark green with a black border. c Effect of tissue and age. Healthy tissue of female patients is represented in orange without a border; Tumour tissue of female patients is represented in red with a black border; Healthy tissue of male patients is represented in light blue without a border; Tumour tissue of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

AQPs' relative expression is not known to be gender dependent. In this study, it was observed a significant variation in AQP1, decreasing in both genders in tumour tissues. AQP1 expression decreases 80.3 % in the female patients (HT = 1.47; TT = 0.289), while decreasing 68.8 % in the male patients (HT = 0.776; TT = 0.242). Regarding the differences between the genders, AQP1 expression is not different between male and female patients in healthy or tumour tissues. AQP1 expression is indeed significantly different between healthy tissues and tumour tissues in female patients (P = 0.007). AQP3, AQP5, and AQP9 relative expressions were not found to be significantly different between female and male patients (P > 0.05).

4.2.2. E-cadherin and Vimentin

E-cadherin and Vimentin relative expression is not age- or gender-dependent. All results are represented in figure 10.

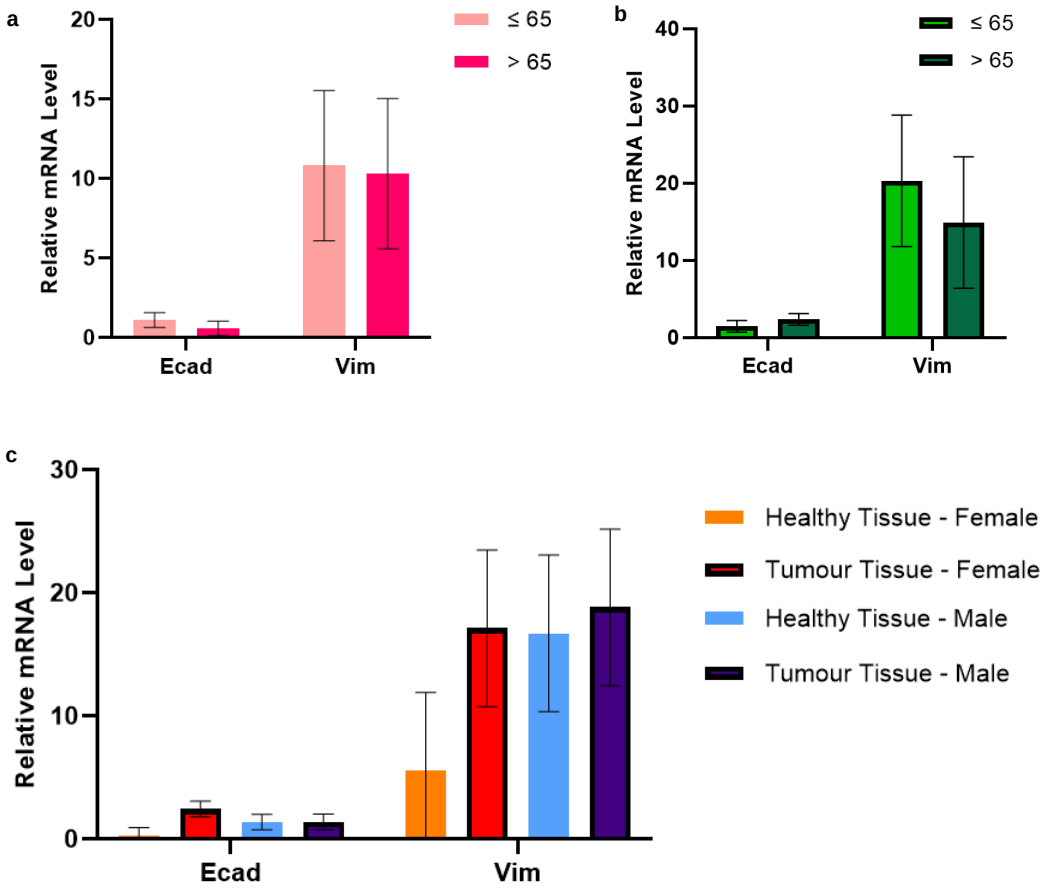

Figure 10 - Effect of tissue, age, and gender on the relative gene expression of Ecad and Vim. a Effect of age in healthy tissues. The Young Group (≤ 65 years old) are represented in light rose without a border; the Old Group (> 65 years old) is represented in dark rose without a border. b Effect of age in tumour tissues. The Young Group (≤ 65 years old) are represented in light green with a black border; the Old Group (> 65 years old) is represented in dark green with a black border. c Effect of tissue and age. Healthy tissue of female patients is represented in orange without a border; Tumour tissue of female patients is represented in red with a black border; Healthy tissue of male patients is represented in light blue without a border; Tumour tissue of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

In healthy tissues, Ecad and Vim relative expression decreased in the Old Group, 48.2 % and 4.63 % respectively. These results are not statistically significant (P > 0.05). In tumour tissues, the behaviour for Ecad relative expression changes, increasing 62.1 % in the Old Group, while Vim relative expression decreases by 26.6 %. These results are also not statistically significant (P > 0.05).

Regarding gender, Vim and Ecad relative expression increased in both tumour tissues of female and male patients. Nevertheless, the values are not significantly different (P > 0.05).

4.2.3. EGFR and ERK MAPK Pathway Proteins

In healthy tissues, EGFR, ERK1, ERK2, c-Jun, and c-Fos relative expression is not age-dependent. However, there is an increase of ERK1 relative expression in the Old Group healthy tissues by 49.7, which is not statistically significant (P = 0.552).

This behaviour shifts in the tumour tissues, and ERK1 relative expression decreases significantly in the Old Group by 77 % (Young Group = 13.1; Old Group = 3.01; P = 0.042). The dynamic is observable in the other signalling pathway components, ERK2, c-Jun, and c-Fos, with their relative expression also decreasing (41.3 %, 46.5 %, and 60.6 % respectively), but the differences are not statistically significant (P > 0.05).

Regarding gender, the genes' relative expression does not variate accordingly. All differences are not statistically significant (P > 0.05). All results are represented in figure 11.

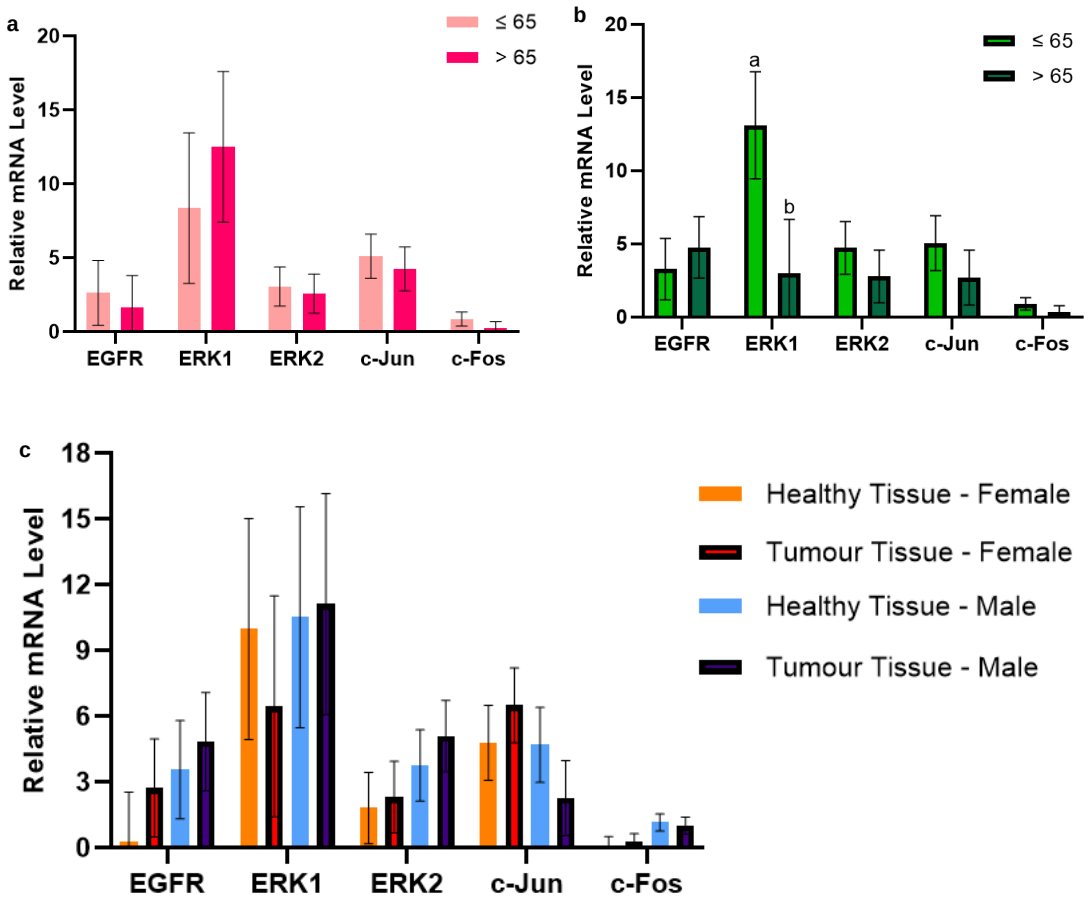

Figure 11 - Effect of tissue, age, and gender on the relative gene expression of EGFR, ERK1, ERK2, c-Jun, and c-Fos. a Effect of age in healthy tissues. The Young Group (≤ 65 years old) are represented in light rose without a border; the Old Group (> 65 years old) is represented in dark rose without a border. b Effect of age in tumour tissues. The Young Group (≤ 65 years old) are represented in light green with a black border; the Old Group (> 65 years old) is represented in dark green with a black border. c Effect of tissue and age. Healthy tissue of female patients is represented in orange without a border; Tumour tissue of female patients is represented in red with a black border; Healthy tissue of male patients is represented in light blue without a border; Tumour tissue of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.3. Aggressive Grade

4.3.1. Aquaporins

The involvement of aquaporins in lower and higher aggressive types of tumours was evaluated only considering their relative expression in the tumour tissues. All results are represented in figure 12.

Aquaporins' relative expression was found to be increased in low-aggressive grade tumour tissues. AQP1 was increased by 72.8 % in low-aggressive grade tumour tissues. AQP3 was increased by 66.3 % in low-aggressive grade tumour tissues. AQP5 was increased by 258 % and AQP9 was increased by 1694 % in low-aggressive grade tumour tissues. These results are not statistically significant ($P > 0.05$), however the results for AQP5 and AQP9 deserve attention since it indicates a possible association between AQP5 and AQP9 expressions and cancers with low-aggressive grades.

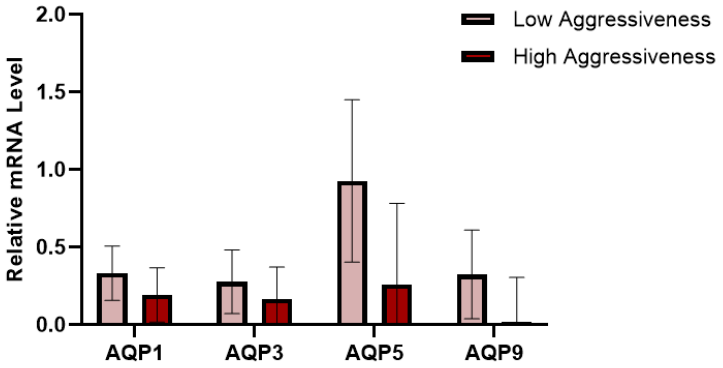

Figure 12 - Effect of aggressive grade on the relative gene expression of AQP1, AQP3, AQP5, and AQP9. Tumour tissues with a low-aggressive grade are represented in light pink with a black border; tumour tissues with a high-aggressive grade are represented in dark red with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, $P < 0.05$).

4.3.2. E-cadherin and Vimentin

E-cadherin and Vimentin have different behaviours in low- and high-aggressive grade tumours. All results are represented in figure 13. Ecad maintains the same expression dynamic, independently of grade (Low-grade = 1.93; High-grade = 1.92). Vim relative expression increases by 171 % in high-aggressive grade tumour tissues (Low-grade = 9.31; High-grade = 25.2). Still, these results were not statistically significant (P > 0.05).

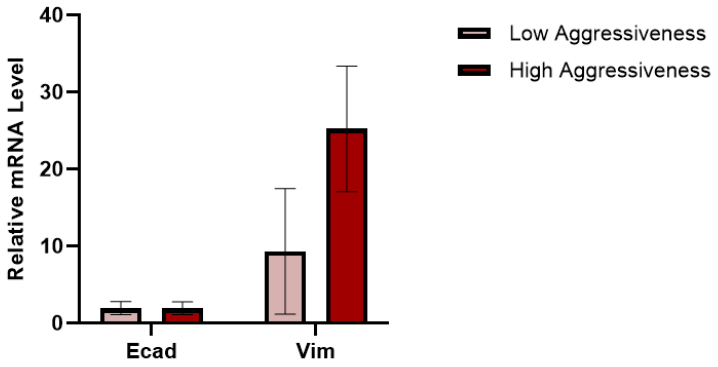

Figure 13 - Effect of aggressive grade on the relative gene expression of Ecad and Vim. Tumour tissues with a low-aggressive grade are represented in light pink with a black border; tumour tissues with a high-aggressive grade are represented in dark red with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.3.3. EGFR and ERK MAPK Pathway Proteins

EGFR, ERK1, ERK2, c-Jun, and c-Fos relative expression follow the same variations detected for the aquaporins relative expression, described before. All results are represented in figure 14.

EGFR relative expression is increased in low-aggressive grade tumour tissues by 396 % (Low-grade = 5.95; High-grade = 1.2). ERK1 relative expression is increased by 64.3 % in low-aggressive grade tumour tissues (Low-grade = 11.5; High-grade = 7). ERK2 relative expression is increased in low-aggressive grade tumour tissues by 128 % (Low-grade = 5; high-grade = 2.19). c-Jun relative expression is increased by 66 % in low-aggressive grade tumour tissues (Low-grade = 4.99; High-grade = 3). Finally, c-Fos relative expression in low-aggressive grade tumour tissues is increased by 597 % (Low-grade = 1.08; High-grade = 0.155). These findings are not statistically significant (P > 0.05).

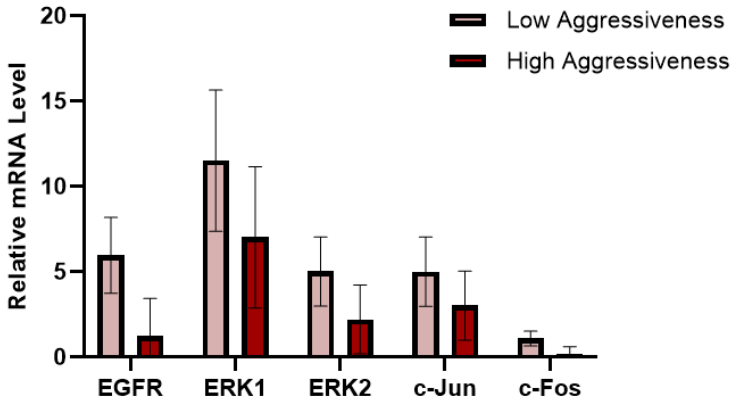

Figure 14 - Effect of aggressive grade on the relative gene expression of EGFR, ERK1, ERK2, c-Jun, and c-Fos. Tumour tissues with a low-aggressive grade are represented in light pink with a black border; tumour tissues with a high-aggressive grade are represented in dark red with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.4. Gender in Aggressive Grade

4.4.1. Aquaporins

Aquaporins' relative expression does not vary significantly ($P > 0.05$) between gender or aggressive grade. Nevertheless, the results show an increase in AQP3, AQP5, and AQP9 relative expression between low-aggressive grade tumour tissues in female patients, which would mean a higher influence on female patients with pancreatic cancer. All results are represented in figure 15.

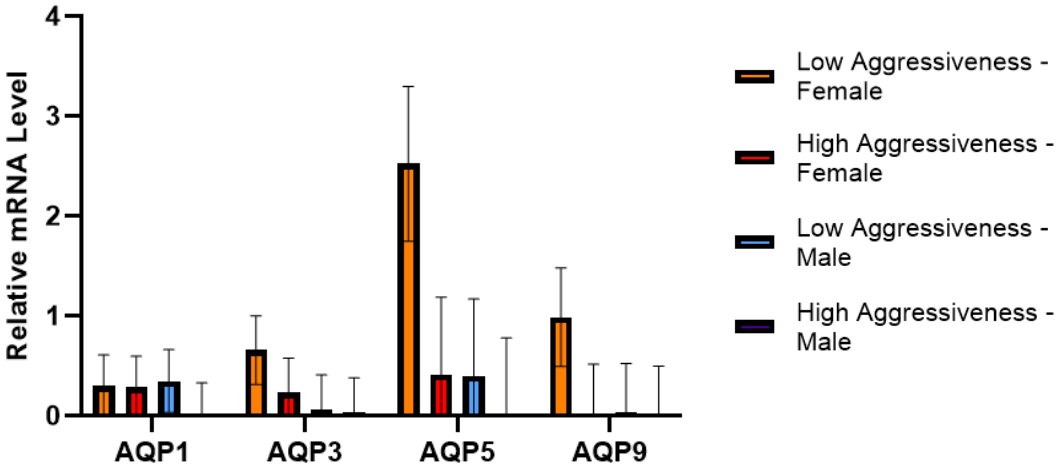

Figure 15 - Effect of aggressive grade and gender on the relative gene expression of AQP1, AQP3, AQP5, and AQP9. Tumour tissues with low-aggressive grade of female patients is represented in orange with a black border; Tumour tissue with high-aggressive grade of female patients is represented in red with a black border; Tumour tissue with low-aggressive grade of male patients is represented in light blue with a black border; Tumour tissue with high-aggressive grade of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, $P < 0.05$).

4.4.2. E-cadherin and Vimentin

The results previously reported in 3.3.2. are observed when analysing Ecad and Vim relative expression by gender. Ecad and Vim present the same variation, with higher values for high-aggressive grade tumour in female patients, increasing when comparing with high-aggressive grade tumour in male patients. However, Vim relative expression is higher in low-aggressive grade tumours in male patients, when comparing with low-aggressive grade tumours in female patients. Ecad follows the opposite tendency. All results are not statistically significant and are presented in figure 16.

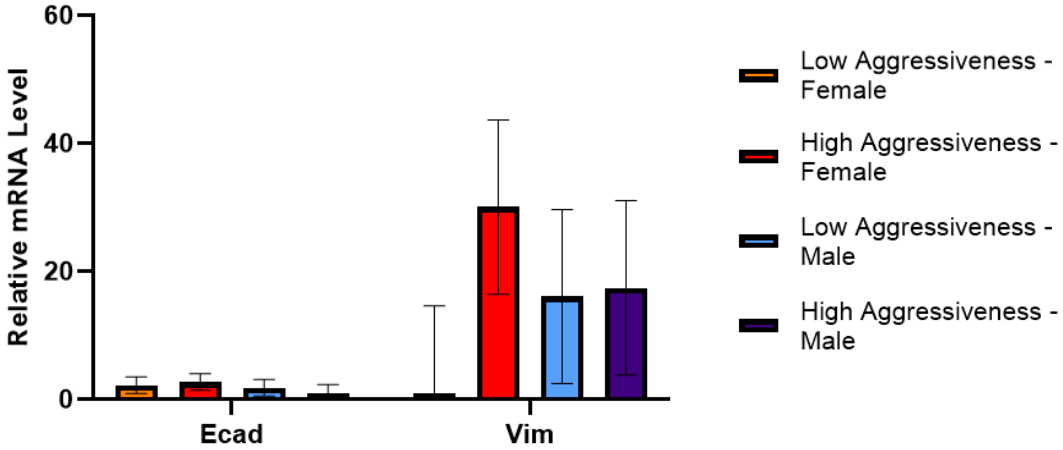

Figure 16 - Effect of aggressive grade and gender on the relative gene expression of Ecad and Vim. Tumour tissues with low-aggressive grade of female patients is represented in orange with a black border; Tumour tissue with high-aggressive grade of female patients is represented in red with a black border; Tumour tissue with low-aggressive grade of male patients is represented in light blue with a black border; Tumour tissue with high-aggressive grade of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.4.3. EGFR and ERK MAPK Pathway Proteins

EGFR, ERK1, ERK2, c-Jun and c-Fos relative gene expression have different patterns. While c-Jun is overexpressed in low-aggressive grade tumours in female patients, EGFR, ERK1, ERK2, and c-Fos are overexpressed in low-aggressive grade tumours in male patients. The results are presented in figure 17.

c-Jun relative expression results are statistically significant, existing a higher expression in low-aggressive grade tumours in female patients than in male patients (P = 0.029).

EGFR, ERK1, ERK2, and c-Fos relative expression results are not statistically significant (P > 0.05). However, since all genes are overexpressed in the same aggressive grade and gender, it is possible that c-Jun, mainly JNK signalling pathway, would be involved in low-aggressive grade tumours in female patients, while ERK signalling pathway, stimulated by EGFR, would be involved in low-aggressive grade tumours in male patients.

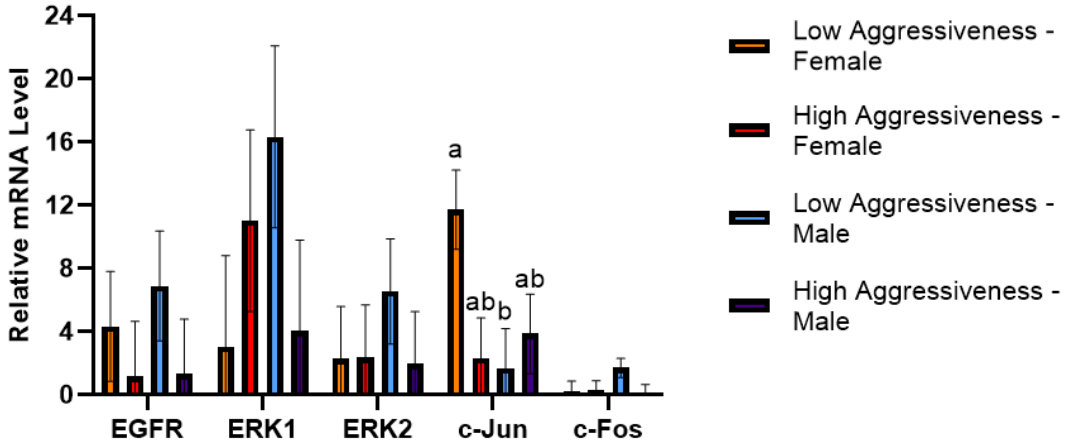

Figure 17 - Effect of aggressive grade and gender on the relative gene expression of EGFR, ERK1, ERK2, c-Jun, and c-Fos. Tumour tissues with low-aggressive grade of female patients is represented in orange with a black border; Tumour tissue with high-aggressive grade of female patients is represented in red with a black border; Tumour tissue with low-aggressive grade of male patients is represented in light blue with a black border; Tumour tissue with high-aggressive grade of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.5. Invasiveness Grade

4.5.1. Aquaporins

The results observed for aquaporins' relative expression regarding invasive grade are very interesting, being presented in figure 18. AQP1 relative expression increases slightly in high-invasive grade tumours, without being significantly different (P > 0.05). AQP3 relative expression, on the other hand, is higher in low-invasive grade tumours than in high-invasive grade tumours by 781 % (Low-grade = 0.608; High-grade = 0.069), which directly associates AQP3 relative expression to low-invasive grade tumours, with statistically significant differences between both grades (P = 0.046). AQP9 relative expression follows the same variation, with higher expression in low-invasive grade tumours by 1263 %, however, these results were not statistically significant (P = 0.090). AQP5 relative expression has a different dynamic, with higher expression being observed in high-invasive grade tumours. This would associate AQP3, and AQP9 with low-invasive grade tumours, while AQP5 would be associated with high-invasive grade tumours. This is not the case, since the results for AQP5 relative expression were not statistically significant (P = 0.550).

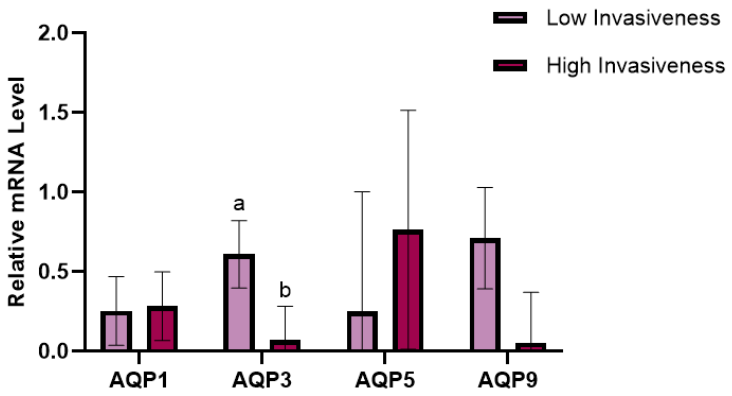

Figure 18 - Effect of invasive grade on the relative gene expression of AQP1, AQP3, AQP5, and AQP9. Tumour tissues with a low-invasive grade are represented in light pink with a black border; tumour tissues with a high-invasive grade are represented in dark red with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.5.2. E-cadherin and Vimentin

E-cadherin and Vimentin relative expression have the same dynamic. Both genes are overexpressed in high-invasive grade tumours. Vimentin relative expression is lower than Ecad in low-invasive grade tumours. These results are not statistically significant (P > 0.05) and are presented in figure 19.

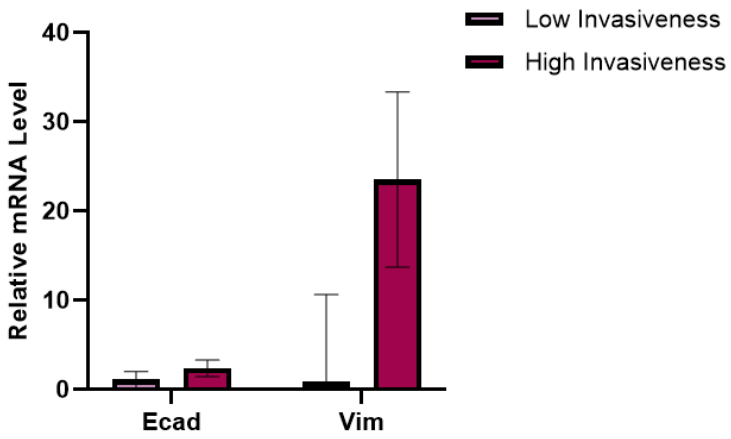

Figure 19 - Effect of invasive grade on the relative gene expression of Ecad and Vim. Tumour tissues with a low-invasive grade are represented in light pink with a black border; tumour tissues with a high-invasive grade are represented in dark red with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.5.3. EGFR and ERK MAPK Pathway Proteins

In low- and high-invasive grade tumours, EGFR, ERK1, ERK2, c-Jun, and c-Fos relative expression have different dynamics. EGFR, ERK2, and c-Fos are overexpressed in high invasive cancer by 355 %, 39.6 %, and 229 % respectively. ERK1, however, is overexpressed in low-invasive cancer by 18.9 %. These results are not statistically significant ($P > 0.05$) but bring up the possibility of ERK pathway being more influential in high-invasive grade tumours.

c-Jun relative expression is higher in low-invasive grade tumours than in high-invasive grade ones. This result is statistically significant ($P = 0.033$), which indicates a higher influence of c-Jun, namely JNK signalling pathway, in low-invasive grade tumours, possibly associated with AQP3 relative expression. All the results are represented in figure 20.

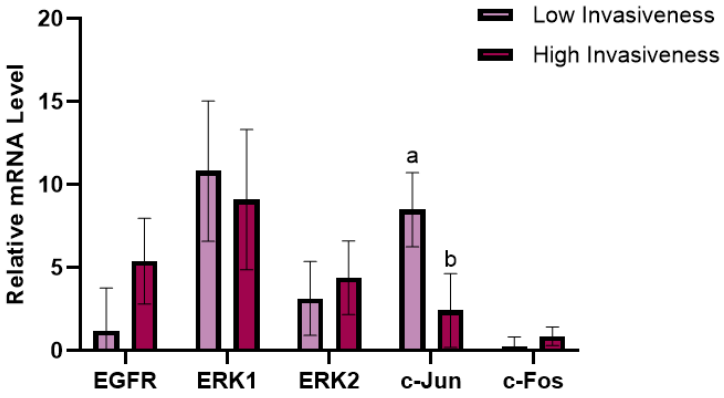

Figure 20 - Effect of invasive grade on the relative gene expression of EGFR, ERK1, ERK2, c-Jun, and c-Fos. Tumour tissues with a low-invasive grade are represented in light pink with a black border; tumour tissues with a high-invasive grade are represented in dark red with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, $P < 0.05$).

4.6. Gender in Invasiveness Grade

4.6.1. Aquaporins

Aquaporins have different relative expressions depending on invasive grade and gender, however none of the results are statistically significant (P > 0.05).

In line with what was already observed in 4.5.1., AQP3 and AQP9 relative expressions are higher in low-invasive grade tumours from female patients, while AQP5 relative expression is higher in high-invasive grade tumours from female patients. AQP5 relative expression in low-invasive grade tumours from female patients is also higher than in low-invasive tumours from male patients, which can also be observed for AQP1 relative expression. This would mean all AQPs are involved in low-invasive grade tumours in female patients, while only AQP5 would be involved in high-invasive grade tumours in female patients. The results are represented in figure 21.

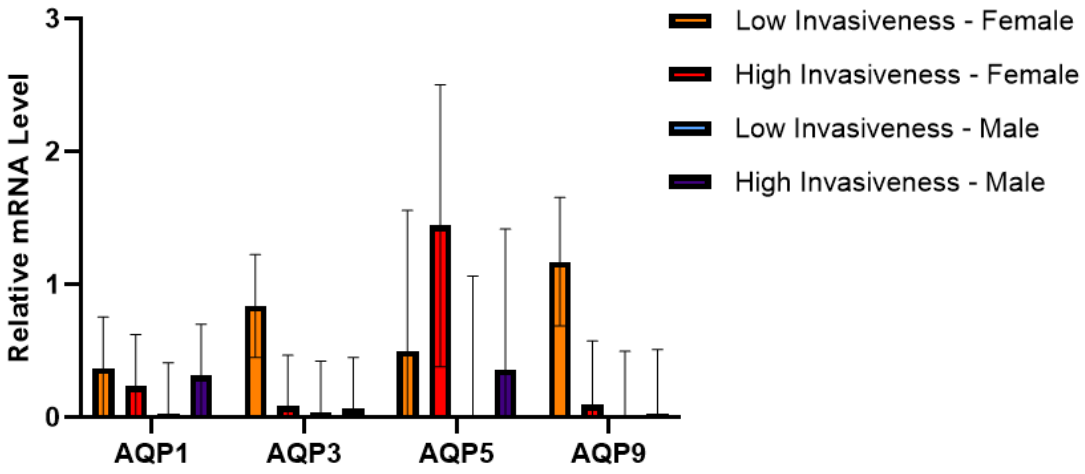

Figure 21 - Effect of invasive grade and gender on the relative gene expression of AQP1, AQP3, AQP5, and AQP9. Tumour tissues with low-invasive grade of female patients is represented in orange with a black border; Tumour tissue with high-invasive grade of female patients is represented in red with a black border; Tumour tissue with low-invasive grade of male patients is represented in light blue with a black border; Tumour tissue with high-invasive grade of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.6.2. E-cadherin and Vimentin

Ecad and Vim are overexpressed in high-invasive grade tumours in female patients, compared with their relative expression values for low-invasive grade tumours in female patients. While Vim relative expression is not statistically different between genders, existing a tendency in high-invasive grade tumours between genders (P = 0.061), Ecad relative expression between high-invasive grade tumours in female patients and male patients is significantly different (P = 0.004). This study did not obtain results for low-invasive grade tumours in male patients for Vim relative expression, which is also very low in low-invasive grade tumours in female patients (low-grade, female patients = 0.806). These results are represented in figure 22.

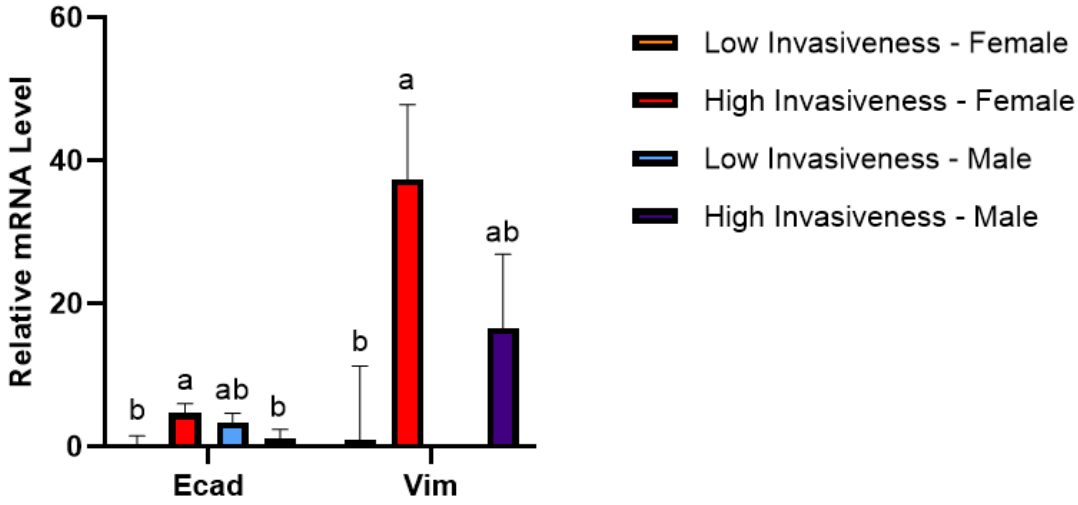

Figure 22 - Effect of invasive grade and gender on the relative gene expression of Ecad and Vim. Tumour tissues with low-invasive grade of female patients is represented in orange with a black border; Tumour tissue with high-invasive grade of female patients is represented in red with a black border; Tumour tissue with low-invasive grade of male patients is represented in light blue with a black border; Tumour tissue with high-invasive grade of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.6.3. EGFR and ERK MAPK Pathway Proteins

When evaluating EGFR, ERK1, ERK2, c-Jun, and c-Fos relative expression regarding invasive grade, the patterns are not very well defined. The results are represented in figure 23.

While EGFR relative expression is higher in high-invasive grade tumours for both genders, without it being statistically significant (P > 0.05), ERK1 and ERK2 relative expression is higher in low-invasive grade tumours. Not only it is higher in this invasive grade, but also higher in male patients comparing with the values for female patients. Regarding ERK1 alone, these results are statistically significant (P = 0.002), indicating an association between grade and gender. ERK2 differences are not statistically significant (P = 0.180). c-Jun on the other hand have higher relative expression values in low-invasive grade in female patients, once again indicating a possible association with pancreatic cancer in female patients, especially in low-invasive grade tumours. However, these results are not statistically significant (P = 0.155). c-Fos has higher relative expression values in male patients than in female patients, both in low- and high-invasive grade, without it being significant (P = 0.579).

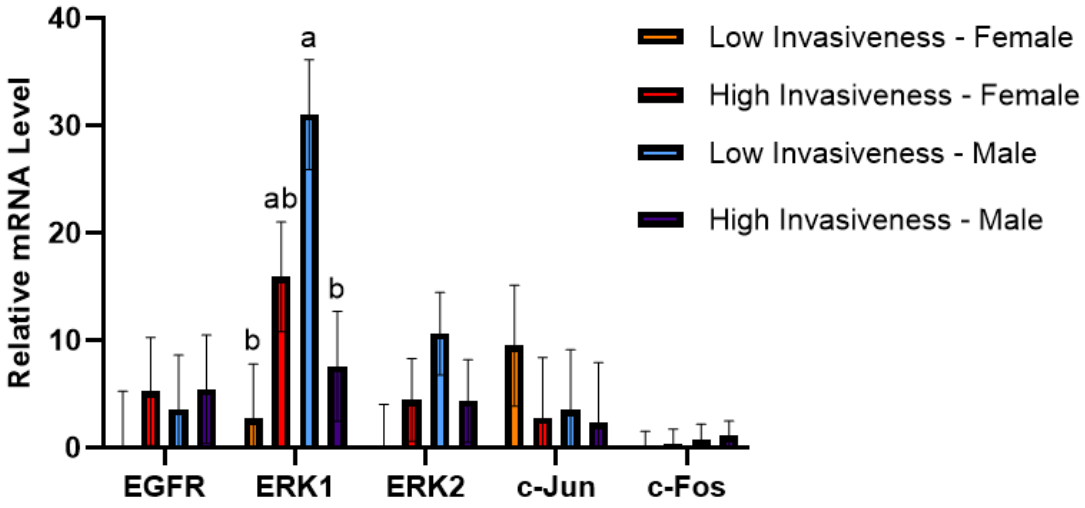

Figure 23 - Effect of invasive grade and gender on the relative gene expression of EGFR, ERK1, ERK2, c-Jun, and c-Fos. Tumour tissues with low-invasive grade of female patients is represented in orange with a black border; Tumour tissue with high-invasive grade of female patients is represented in red with a black border; Tumour tissue with low-invasive grade of male patients is represented in light blue with a black border; Tumour tissue with high-invasive grade of male patients is represented in dark blue with a black border. Values are means with their standard errors represented by vertical bars. Mean values with unlike letters were significantly different (Tukey's *post hoc*, P < 0.05).

4.7. Pearson's Correlations

Figure 24 presents Pearson's correlations coefficients among mRNA expression levels of AQPs, Ecad, Vim, EGFR, ERK1, ERK2, c-Jun, and c-Fos in healthy tissues. All the correlations were found to be positive, high or moderate correlations (high correlation, r > 0.7; moderate correlation, 0.7 ≥ r > ≥ 0.3; low correlation, r < 0.3) (Costa et al., 2012).

In the healthy tissues, AQP3 was found correlated with AQP1 (r = 0.701, P = 0.0004). AQP5 was correlated with AQP1 (r = 0.902, P = 0.0001) and AQP3 (r = 0.647, P = 0.031). AQP9 was correlated with AQP1 (r = 0.859, P = 0.0007), AQP3 (r = 0.986, P < 0.0001), and AQP5 (r = 0.915, P = 0.0015). Vim was correlated with Ecad (r = 0.487, P = 0.041). ERK1 was correlated with Vim (r = 0.505, P = 0.033). ERK2 was correlated with Vim (r = 0.707, P = 0.0015) and ERK1 (r = 0.842, P < 0.0001). c-Fos was correlated with Ecad (r = 0.892, P < 0.0001), Vim (r = 0.742, P = 0.0004), EGFR (r = 0.617, P = 0.008), ERK1 (r = 0.607, P = 0.008), and ERK2 (r = 0.567, P = 0.022).

	AQP1	AQP3	AQP5	AQP9	Ecad	Vim	EGFR	ERK1	ERK2	c-Jun	c-Fos
AQP1	1.00	0.701 0.0004	0.902 0.0001	0.859 0.0007	-0.254 0.310	-0.116 0.648	-0.255 0.307	-0.289 0.244	-0.142 0.588	-0.257 0.273	-0.361 0.141
AQP3		1.00	0.647 0.031	0.986 <.0001	-0.195 0.469	0.038 0.890	-0.196 0.468	-0.094 0.730	-0.118 0.663	-0.108 0.671	-0.213 0.413
AQP5			1.00	0.915 0.0015	-0.325 0.477	-0.294 0.480	-0.293 0.444	-0.332 0.422	-0.203 0.601	0.169 0.664	-0.423 0.344
AQP9				1.00	-0.312 0.452	-0.036 0.932	-0.237 0.511	-0.143 0.694	-0.258 0.473	-0.184 0.611	-0.266 0.489
Ecad					1.00	0.487 0.041	0.397 0.115	0.211 0.401	0.400 0.111	-0.144 0.557	0.892 <.0001
Vim						1.00	0.466 0.059	0.505 0.033	0.707 0.0015	-0.177 0.470	0.742 0.0004
EGFR							1.00	0.135 0.592	0.058 0.826	0.397 0.103	0.617 0.008
ERK1								1.00	0.842 <.0001	0.002 0.994	0.607 0.008
ERK2									1.00	-0.137 0.587	0.567 0.022
c-Jun										1.00	0.020 0.937
c-Fos											1.00

Figure 24 - Pearson's correlations among mRNA expression levels of AQPs, Ecad, Vim, EGFR, ERK1, ERK2, c-Jun, c-Fos in the healthy tissues. The first number on the square is the value of Pearson correlation coefficient (r) and the number blow is the P value for each correlation.

Figure 25 presents Pearson's correlations coefficients among mRNA expression levels of AQPs, Ecad, Vim, EGFR, ERK1, ERK2, c-Jun, and c-Fos in tumour tissues. All the correlations were found to be positive, high or moderate correlations (the coefficient values for the correlation to be considered high, moderate or low, were explained earlier in the text).

In the tumour tissues, AQP9 was found correlated with AQP3 ($r = 0.992$, $P < 0.0001$) and AQP 5 ($r = 0.975$, $P < 0.0001$). Ecad was correlated with AQP5 ($r = 0.737$, $P = 0.0005$). EGFR was correlated with AQP5 ($r = 0.457$, $P = 0.043$) and Ecad ($r = 0.744$, $P < 0.0001$). ERK1 was correlated with Ecad ($r = 0.689$, $P = 0.001$) and Vim ($r = 0.719$, $P = 0.006$). ERK2 was correlated with ERK1 ($r = 0.796$, $P < 0.0001$). c-Jun was correlated with AQP3 ($r = 0.852$, $P < 0.0001$) and AQP9 ($r = 0.952$, $P < 0.0001$). c-Fos was correlated with ERK1 ($r = 0.567$, $P = 0.014$) and ERK2 ($r = 0.667$, $P = 0.001$).

	AQP1	AQP3	AQP5	AQP9	Ecad	Vim	EGFR	ERK1	ERK2	c-Jun	c-Fos
AQP1	1.00	0.170 0.438	0.073 0.752	0.132 0.581	-0.235 0.319	-0.051 0.850	-0.145 0.520	-0.238 0.357	-0.255 0.266	0.282 0.228	-0.239 0.311
AQP3		1.00	0.383 0.087	0.992 <.0001	-0.210 0.361	-0.287 0.265	-0.128 0.562	-0.202 0.421	-0.212 0.343	0.852 <.0001	-0.083 0.721
AQP5			1.00	0.975 <.0001	0.737 0.0005	-0.064 0.827	0.457 0.043	-0.334 0.223	0.148 0.546	0.158 0.532	-0.121 0.631
AQP9				1.00	-0.124 0.625	-0.272 0.347	-0.092 0.698	-0.185 0.508	-0.144 0.545	0.952 <.0001	-0.049 0.848
Ecad					1.00	0.441 0.100	0.744 <.0001	0.689 0.001	0.395 0.076	-0.320 0.169	0.063 0.787
Vim						1.00	-0.073 0.781	0.719 0.006	0.361 0.170	-0.237 0.359	0.066 0.808
EGFR							1.00	0.259 0.285	0.098 0.658	-0.239 0.284	0.150 0.506
ERK1								1.00	0.796 <.0001	-0.192 0.445	0.567 0.014
ERK2									1.00	-0.217 0.345	0.667 0.001
c-Jun										1.00	-0.153 0.508
c-Fos											1.00

Figure 25 - Pearson's correlations among mRNA expression levels of AQPs, Ecad, Vim, EGFR, ERK1, ERK2, c-Jun, c-Fos in the tumour tissues. The first number on the square is the value of Pearson correlation coefficient (r) and the number blow is the P value for each correlation.

5. Discussion

This study hypothesised that aquaporins are involved in pancreatic cancer and are correlated with cancer signalling pathways such as the ERK MAPK pathway and EMT. All the genes studied (AQP1, AQP3, AQP5, AQP9, Ecad, Vim, ERK1, ERK2, c-Jun, and c-Fos) were found expressed in both tumour and healthy pancreatic tissues.

AQP3, AQP5, and AQP9 were not found overexpressed in the tumour tissues under analysis, while AQP1 presented a relative expression decreased. This result does not corroborate the initial hypothesis, since AQP1 was observed to be overexpressed in tumour tissues previously (Zou et al., 2019), and thought as a possible biomarker for pancreatic cancer. The research on AQP1 expression and its involvement in this specific cancer type is needed to understand the possible correlation between AQP1 and pancreatic cancer. Nevertheless, AQP3, AQP5 and AQP9 seem to have a role in pancreatic cancer, even if not overexpressed. AQP9 relative expression is, to the best of our knowledge, for the first time identified in pancreatic healthy and tumour tissues.

All aquaporins relative expression was higher in low-aggressive grade tumours than in high-aggressive grade tumours, which was observed also for the EGFR and ERK MAPK proteins. If these results were statistically significant, this would indicate a possible relationship between AQPs' expression and EGFR and ERK MAPK cascade activation, observed in colon and lung cancer, and glioma cells (Li et al., 2018; Shi et al., 2014; Yang et al., 2017). Specifically AQP5 relative expression results would be align with the results obtained for PDAC, where AQP5 was identified and correlated with moderately differentiated tumours, in other words, with low-aggressive grade tumours (Direito et al., 2017).

On the other side, AQP3 and c-Jun have higher relative expression values for low-invasive grade tumours than in high-invasive grade tumours. This is surprising, since AQP3 is normally associated with more aggressive and invasive cancers. Different studies have determined AQP3 as involved in increased migration and invasion of breast cancer cells (Huang et al., 2015; Satooka & Hara-Chikuma, 2016), and invasiveness in prostate cancer cells (Chen et al., 2015). This result is interesting, because not only indicates AQP3 as a possible biomarker for invasiveness, specifically for low-invasive grade tumours, but it confirms the Pearson's correlation obtained between AQP3 and c-Jun. AQP3 and JNK MAPK interplay was already identified in keratinocytes cell culture, where JNK MAPK pathway was involved in AQP3 downregulation (Bae et al., 2019); in human colon epithelial cell culture, in which AQP3 was downregulated by lipopolysaccharide via p38/JNK MAPK signalling pathway (Li et al., 2015); and in human amnion epithelium cells, where AQP3 was downregulated through JNK MAPK signalling activation (Wang et al., 2014). This is, therefore, the first time AQP3 and c-Jun were correlated positively with each other in pancreatic cancer. c-Jun expression was associated with cell cycle progression and apoptosis inhibition (Wisdom et al., 1999), which may reflect the role of AQP3 in this tumour type.

Vimentin was overexpressed in high-aggressive and high-invasive grade tumours, while Ecad is still expressed in tumour tissues. This was the most surprising result; however, it can be explained since the tumours were in different stages of differentiation, therefore in different stages of EMT (Huang et al., 2013; Jolly et al., 2016; Pastushenko et al., 2018). Furthermore, in a study regarding EMT in pancreas (Kohler et al., 2015), Ecad was observed to still be expressed in tumour tissues. Ecad was differently expressed in regions of tumours, decreasing toward the tumour periphery. Ecad expression in the nuclear region was also associated with other signalling pathway, Wnt/β-catenin, which was not addressed in this study.

Aquaporins expression was not influenced by age or gender, nor influenced by aggressiveness and invasiveness grades. However, Vim, Ecad and the ERK MAPK signalling pathway presented some interesting variations.

Ecad, for instance, had higher relative expression values in high-invasive grade tumours of female patients than of male patients, while Vim presented the same tendency for high-invasive grade tumours of female patients than of male patients. Combined, Ecad and Vim could be used as biomarkers of poor-prognosis and invasiveness on female patients with pancreatic cancer. In a study focused on other EMT biomarkers, 9 out of 16 genes showed different values of hazard ratio depending on gender in hepatocellular cancer, demonstrated that gender might affect the regulation of EMT-related gene expression, therefore, playing a role in cancer malignancy, and consequently shortening overall survival (Song & Kim, 2019).

On the other hand, ERK1 mRNA levels were increased in the Young Group compared with the Old Group in tumour tissues, which is a change from the dynamic observed in healthy tissues, with the Old Group with higher ERK mRNA levels than the Young Group. This result implies a significant disturb in ERK MAPK on the Old Group, with ERK2, c-Jun and c-Fos also decreasing mRNA levels. This variation dependent on age was never described for cancer. ERK1 was also overexpressed in low-invasive grade tumours in male patients. The difference between male and female patients was statistically significant, with the same behaviour being observed for ERK2. Also, the difference between low- and high-invasive grade for male patients was statistically significant, which indicates a possibility of ERK1 representing a biomarker for invasiveness in male patients. c-Jun was also observed to be gender variable, with higher expression in low-aggressive grade tumour in female patients when compared with male patients. However, c-Jun didn't present significant differences between grades in either female or male patients and isn't useful as a biomarker for aggressiveness. However, this indicates that c-Jun is stimulated in female patients. A study showed that certain sex hormones can inhibit inflammatory and prop-apoptotic processes in kidney diseases (Grzegorczyk et al., 2011), with female patients presenting a protective factor towards disease. Nevertheless, the study also determined that after menopause this protective effect is no longer observed. Since most of the female patients in this case were already on or entering menopause (considering the patients were older than 50 years old), this protective effect shouldn't be considered.

All these results and conclusions need confirmation and further validation. Regarding specifically ERK MAPK and JNK MAPK pathways, this information is not present to our best knowledge in the literature, regarding pancreatic or other cancer type. However, the suggestion of specifically ERK being overexpressed in an age group and gender would help consider different approaches of treatment, depending on its prognostic value.

For last, evaluating the Pearson's correlations it's possible to observe that in healthy tissues, aquaporins all correlate to each other. AQP1 and AQP5 are classical aquaporins, AQP3 and AQP9 are aquaglyceroporins, and all these aquaporins are peroxiporins. Since there is a need for aquaporins' functions in the pancreas to guarantee the well physiological function, it is natural that aquaporins stimulate each other's relative expression, especially in conditions when water permeability increases (Kachadorian et al., 2000).

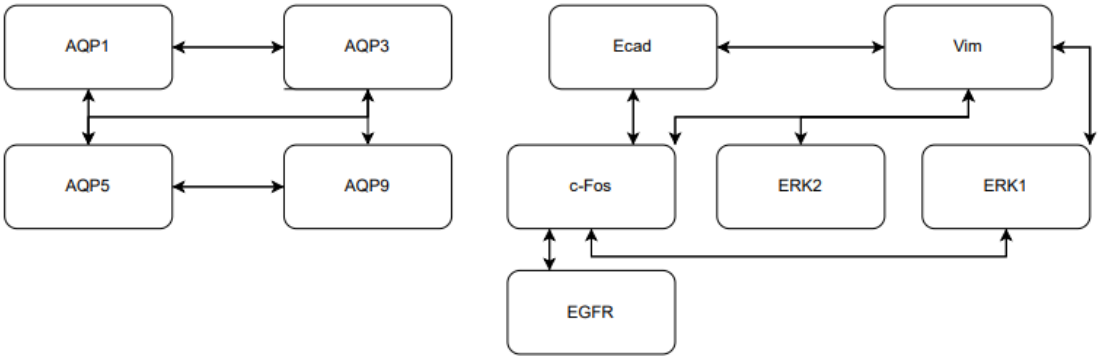

Figure 26 - Diagram of Pearson's Correlations in Healthy Tissues.

Aquaporins do not correlate with ERK MAPK signalling pathway in healthy tissues. Interestingly, this reality completely changes when the cell suffers a transformation and becomes a cancer cell, disrupting the normal function of the cell and hyperactivating signalling pathways (Ullah et al., 2021).

In tumour tissues, AQP3 only correlates with AQP9, while AQP9 correlates both with AQP3 and AQP5. But the interesting result is that both AQP3 and AQP9 correlate with c-Jun, offering a direct association between AQP3 and AQP9 mRNA levels and c-Jun mRNA levels. Therefore, AQP3 and AQP9 are either influenced by or influence JNK MAPK. At the same time, AQP5 is correlated with EGFR and Ecad, which is correlated with ERK1 and c-Fos. These results suggest that AQP5 influences or is influenced by EGFR and ERK MAPK signalling pathway indirectly. AQP5 is also involved in EMT positively, engaging with Ecad. These correlations are illustrated in figures 26 and 27.

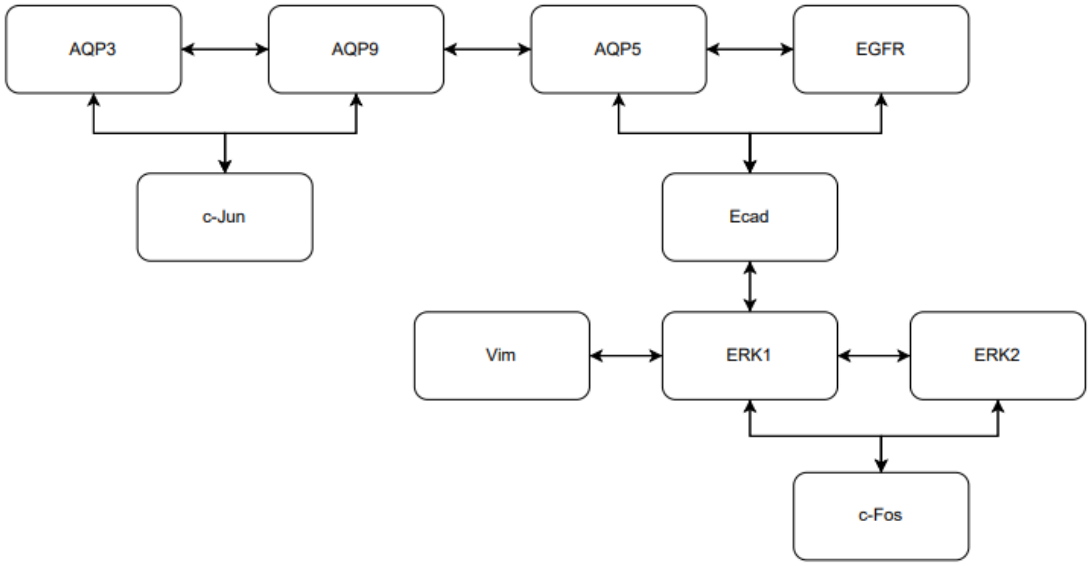

Figure 27 - Diagram of Pearson's Correlation in Tumour Tissues.

6. Conclusion

The use of human tissue samples is very useful to elucidate the tissue-specific variations of proteins and signalling pathways, as well as stimulating factors. However, these variations will produce results with great differences. The larger the cohort, the higher the change to produce statistically significant conclusions.

In this study it was possible to detect the mRNA expression of all genes of interest, specifically AQP1, AQP3, AQP5, and AQP9. However, AQP1 mRNA expression was lower in tumour tissues, and Vimentin was expressed in healthy tissues, contradicting the known literature. AQP9 was identified for the first time in pancreatic healthy and tumour tissues. These discrepancies may be the result of analysing cancer tissues from different pathologic sub-grades, although all diagnosed as pancreatic cancer. Further research with a larger number of samples from each sub-pathology is needed to complement the herein reported data.

Nevertheless, the research found some interesting results. Ecad and Vim mRNA levels were both increased in high-invasive grade tumours in female patients, with Ecad values being statistically significant, and Vim tendentiously. These new findings open the possibility of Ecad and Vim being prognosis biomarkers for invasiveness grade in female patients.

Another molecule which could be useful in prognosis is ERK1, which not only its mRNA levels decreased in tumour tissues from older patients but is overexpressed in low-invasive grade tumours from male patients. Since the difference from low- to high-invasive grade tumour is statistically significant, ERK1 can also serve as a prognosis biomarker for invasiveness grade in male patients.

c-Jun and AQP3 statistically significant results in invasiveness grades, being both overexpressed in low-invasive grade tumours, reflect a possible correlation, that elucidates the effect AQP3 has on cancer, specifically regarding cell proliferation and cell survival. c-Jun and AQP3 can also be used as prognosis biomarkers for invasiveness grade.

The results of the aquaporins expression studied in these experimental conditions indicates that AQP1, AQP3, AQP5, and AQP9 cannot be used as diagnosis biomarkers for pancreatic cancer.

Finally, with Pearson's Correlations, it was possible to draw a new suggestion regarding aquaporins' involvement with signalling pathways. This study has observed the possibility of AQP3 and AQP9 being directly involved with JNK MAPK pathway, and an indirect influence of AQP5 in ERK MAPK pathway and EMT.

These findings need confirmation and validation, with cohorts of bigger dimensions, accompanied by animal models' studies. Nevertheless, we were able to: evaluate and confirm AQP3 as a possible prognosis biomarker in pancreatic cancer and elucidate the interplay of AQPs with JNK MAPK and ERK MAPK signalling pathways, accomplishing the proposed objectives of this thesis.

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