

UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA



**Aquaporins in Metabolic Syndrome:
New Targets for Drug Discovery**

Inês Margarida Vieira da Silva

Orientadores:

- Prof^ª. Doutora Maria da Graça Tavares Rebelo de Soveral Rodrigues
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Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia, especialidade
em Bioquímica

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Instituição financiadora: Fundação para a Ciência e Tecnologia (FCT PD/BD/113634/2015)

Inês Margarida Vieira da Silva foi bolsreira de doutoramento no âmbito do Programa Doutoral “PhD Programme in Medicines and Pharmaceutical Innovation (i3DU)” da Faculdade de Farmácia da Universidade de Lisboa, financiado pela Fundação para a Ciência e Tecnologia do Ministério da Educação e Ciência, Bolsa FCT PD/BD/113634/2015.

FCT

Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR



i3du

De acordo com o disposto no artigo 24º do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa, Despacho nº 7024/2017, publicado no Diário da República – 2ª Série – nº 155 – 11 de Agosto de 2017, foi utilizada nesta dissertação informação incluída nas seguintes publicações:

[2017] **da Silva, I.V.** and Soveral, G. Chapter XV: Aquaporins in Obesity. In Aquaporins, Springer Book Series Subcellular Biochemistry, Baoxue Yang Ed., Adv Exp Med Biol, Springer, Berlin, 969. DOI: 10.1007/978-94-024-1057-0_15

[2017] Mendez-Gimenez, L., Becerril, S., Camoes, S. P., **da Silva, I.V.**, Rodrigues, C., Moncada, R., Valenti, V., Catalan, V., Gomez-Ambrosi, J., Miranda, J.P., Soveral, G., Fruhbeck, G., Rodriguez, A. Role of aquaporin-7 in ghrelin- and GLP-1-induced improvement of pancreatic beta-cell function after sleeve gastrectomy in obese rats, Int J Obes (Lond) 41: 1394-1402. DOI: 10.1038/ijo.2017.135

[2018] **da Silva, I.V.***, Barroso, M.*, Moura, T., Castro, R., Soveral, G. Endothelial Aquaporins and Hypomethylation: Potential Implications for Atherosclerosis and Cardiovascular Disease. Int J Mol Sci 19(1) pii: E130. DOI: 10.3390/ijms19010130

*Equal contribution.

[2018] **da Silva, I.V.**, Rodrigues, J., Rebelo, I., Miranda, J., Soveral, G. Revisiting the Metabolic Syndrome: the emerging role of aquaglyceroporins. Cellular and Molecular Life Sciences 75(11):1973-198. DOI: 10.1007/s00018-018-2781-4

[2018] Mendez-Gimenez, L., Ezquerro, S., **da Silva, I.V.**, Soveral, G., Fruhbeck, G., Rodriguez, A. Pancreatic aquaporin-7: a novel target for anti-diabetic drugs? Frontiers Chemistry 6:99. DOI: 10.3389/fchem.2018.00099

[2018] Lopes, P. A., Martins, R.*, **da Silva, I.V.***, Madeira, M. S., Prates, J. A. M., Soveral, G. Modulation of aquaporin gene expression by n-3 long-chain PUFA lipid structures in white and brown adipose tissue from hamsters. British Journal of Nutrition 120(10):1098-1106. DOI: 10.1017/S0007114518002519

*Equal contribution.

Outras publicações provenientes de trabalho desenvolvido durante o período de desenvolvimento da tese:

[2018] Gomes, A., **da Silva, I.V.**, Rodrigues, C. M. P., Castro, R. E., Soveral, G. The emerging role of microRNAs in aquaporin regulation. Frontiers Chemistry 6:238. DOI: 10.3389/fchem.2018.00238

[2019] Rodrigues, C., Pimpão, C., Mósca, A. F., Coxixo, A. S., Lopes, D., **da Silva, I. V.**, Pedersen, P. A., Antunes, F. Soveral, G. Human Aquaporin-5 Facilitates Hydrogen Peroxide Permeation Affecting Adaption to Oxidative Stress and Cancer Cell Migration. Cancers 11(7): 932. DOI:10.3390/cancers11070932

No cumprimento do disposto da referida deliberação, a autora esclarece serem da sua responsabilidade, a execução das experiências que permitiram a elaboração dos resultados apresentados, assim como da interpretação e discussão dos mesmos. Resultados obtidos por outros autores e incluídos nas publicações são também aqui apresentados para facilitar a compreensão dos trabalhos.

*"The important thing is to never stop questioning.
Curiosity has its own reason for existing."*

Albert Einstein

Aos meus pais

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Summary

Obesity is one of the most important metabolic disorders of this century and is associated with a cluster of the most dangerous cardiovascular disease risk factors, such as insulin resistance, diabetes, dyslipidaemia and hypertension, collectively named Metabolic Syndrome. With the discovery of aquaporins (AQPs, membrane protein channels that facilitate water permeation through membranes) new targets for drug development influenced the research in this field. For now, thirteen aquaporins have been described in humans, whose isoforms 3, 7, 9 and 10 are subcategorized as aquaglyceroporins allowing both water and glycerol transport. The role of aquaporins in glycerol metabolism, facilitating glycerol release from the adipose tissue and distribution to various tissues and organs, unveils these membrane channels as important players in lipid balance and energy homeostasis and points to their involvement in a variety of disorders including insulin resistance, obesity and diabetes.

The thesis introduction (**Chapter 1**) gives an overview on Metabolic Syndrome and Obesity and introduces several structural and physiological aspects of aquaporins that make these proteins potential targets in the context of metabolic health and disease. This chapter describes the aquaporin family and structure, their main biological functions and related pathologies, with special emphasis on aquaglyceroporins and their role as glycerol channels.

The first part of this thesis (**Chapter 2**), is dedicated to the involvement of several mammalian aquaporin isoforms (with focus on aquaglyceroporins) in various obesity-related pathologies, using different types of cells (endothelial cells, pancreatic β -cells and macrophages) as models of study. Using molecular, biochemical and biophysical techniques, we were able to disclose the involvement of aquaporin isoforms in metabolic-related complications, such as endothelial dysfunction, pancreatic β -cell inflammation and inflammasome activation in macrophages.

In the second part of this thesis (**Chapter 3**), AQP3 genetic polymorphisms analysis showed to be a potential tool to predict hypertension. In this study, the analysis of aquaporin isoforms (AQP3 and AQP7) polymorphisms and other relevant hypertension-related gene polymorphisms were evaluated along with biochemical parameters, in a well characterized cohort of pregnant women that were then followed-

up for several years, and were found related to the development of preeclampsia and/or future hypertension.

In the third part of this thesis (**Chapter 4**), the transcriptional profile of adipose aquaporins (aquaglyceroporins AQP3 and AQP7; orthodox aquaporin AQP5) was assessed in white (subcutaneous and visceral) and brown adipose tissue of hamsters fed diets enriched in omega-3 fatty acids (n-3 long chain PUFA) in different lipid structures (triacylglycerol and ethyl ester). A correlation of AQPs differential expression with the type of n-3 lipid structure was found, with the adipose AQP7 showing a conservative role across WAT and BAT with highest targeting potential.

In the last part of the thesis (**Chapter 5**), a general conclusion and final remarks unveil aquaporins as potential targets in the development of new therapies.

Keywords: aquaporins, aquaglyceroporins, metabolism, obesity, inflammation, metabolic syndrome

Resumo

A obesidade é uma das mais importantes doenças metabólicas do século e está associada a um conjunto de fatores de risco fortemente relacionados com a doença cardiovascular, tais como resistência à insulina, diabetes, dislipidemia e hipertensão, geralmente designados em conjunto como Síndrome Metabólica. A descoberta das aquaporinas (AQPs, canais proteicos que facilitam o transporte de água através das membranas celulares) como novos alvos para desenvolvimento de fármacos, influenciou a investigação neste campo. Até agora, foram descritas no Homem treze aquaporinas, classificadas em três grupos de acordo com a sua sequência primária, localização celular e seletividade, 1) aquaporinas ortodoxas, principalmente seletivas para água; 2) aquagliceroporinas, que para além de água também transportam pequenos solutos neutros, como glicerol e ureia; e 3) S-aquaporinas, localizadas em membranas intracelulares e com menor homologia. Devido à grande diversidade de tecidos onde são encontradas as aquaporinas, o seu papel como facilitadoras do transporte de água e/ou solutos através das membranas plasmáticas é importante em vários processos fisiológicos, tais como: secreção de fluido glandular, mecanismo de concentração urinária, metabolismo dos lípidos, hidratação epidérmica e balanço de água no cérebro. A observação do fenótipo de ratinhos geneticamente modificados com silenciamento de certas aquaporinas, revelou funções fisiológicas muito importantes e a sua contribuição para o desenvolvimento de várias patologias, tais como doença renal, edema cerebral, glaucoma, cancro e obesidade. O papel das aquaporinas no metabolismo do glicerol, facilitando o efluxo de glicerol do tecido adiposo e a sua distribuição pelos vários tecidos e órgãos, mostra que estes canais membranares são elementos importantes no equilíbrio lipídico e na homeostasia energética e aponta para o seu envolvimento em várias patologias, incluindo resistência à insulina, obesidade e diabetes.

No **Capítulo 1** é apresentada uma introdução geral que visa proporcionar um conhecimento abrangente sobre o Síndrome Metabólico e a obesidade, mas também introduz vários aspetos sobre as aquaporinas que fazem com que estas proteínas sejam valiosos alvos de estudo num contexto de saúde e doença metabólica. Este capítulo apresenta a família das aquaporinas e descreve a sua estrutura geral, as suas principais funções biológicas e patologias associadas, dando especial atenção às aquagliceroporinas e ao seu papel fundamental como canais de glicerol.

A primeira parte dos resultados desta tese (**Capítulo 2**) é dedicada ao envolvimento de algumas isoformas de aquaporinas de mamíferos (focando maioritariamente aquagliceroporinas) em várias patologias relacionadas com a obesidade, usando diferentes tipos celulares (células endoteliais, células- β pancreáticas e macrófagos) como modelos de estudo. Elucidámos o envolvimento de isoformas de aquaporinas específicas em complicações metabólicas, tais como disfunção endotelial, inflamação do pâncreas endócrino e ativação do processo inflamatório em macrófagos, usando técnicas moleculares, bioquímicas e biofísicas.

Na segunda parte dos resultados desta tese (**Capítulo 3**) a análise da distribuição dos polimorfismos da AQP3 mostrou-se uma ferramenta com potencial para prever o desenvolvimento da hipertensão. Neste capítulo, dedicado ao estudo dos polimorfismos com maior prevalência na população europeia de várias isoformas de aquaporinas com potencial envolvimento em doenças metabólicas (AQP3 e AQP7), e a relacionar a sua prevalência com o desenvolvimento de hipertensão no futuro usando como grupo de estudo uma cohort bem caracterizada de mulheres grávidas seguidas por especialistas por vários anos, foram também avaliados polimorfismos de outros genes com potencial relevância para o desenvolvimento da hipertensão em paralelo com vários parâmetros bioquímicos relevantes.

Na terceira parte dos resultados desta tese (**Capítulo 4**), avaliou-se o perfil transcricional de aquaporinas (aquagliceroporinas AQP3, AQP7 e AQP9; aquaporina ortodoxa AQP5) no tecido adiposo branco (subcutâneo e visceral) e castanho de hamsters alimentados com dietas enriquecidas em ácidos gordos ómega-3 (ácidos gordos polinsaturados n-3 de cadeia longa, EPA E DHA) na forma de diferentes estruturas lipídicas (triacilglicerol e éster etílico). Neste estudo estabeleceu-se uma correlação entre a expressão diferencial das aquaporinas e o tipo de estrutura lipídica em n-3 usada como suplemento na dieta. A AQP7 mostrou ter um papel conservado nos vários tecidos, apresentando maior potencial como alvo de novas terapias.

Para finalizar (**Capítulo 5**), são feitas algumas considerações finais e uma conclusão geral, em que as aquaporinas são consideradas potenciais alvos para o desenvolvimento de novas terapias.

Palavras-chave: aquaporinas, aquagliceroporinas, metabolismo, obesidade, inflamação

General aims and outline of this thesis

In the last decade, the aquaporin field became a very hot area of research in molecular cell biology, biochemistry and biophysics, with increasing physiological and medical implications. Human diseases caused by aquaporin dysfunction (called aquaporinopathies) unveiled these proteins as potential drug targets and opened new perspectives to untangle mechanisms of disease. Thus, knowledge of the regulation of AQP's expression and activity has been considered of major importance in the development of new targeted therapies. Among the AQP's sub-groups, aquaglyceroporins are emerging as important players in adipose tissue homeostasis and insulin response with possible implications in metabolic disorders such as obesity and, consequently, metabolic syndrome.

This thesis is organized in five chapters. In **Chapter 1**, a general introduction of Metabolic Syndrome and Obesity is presented and the state-of-art on aquaporins and their involvement metabolism and metabolic-related complications is stated.

Chapter 2 is divided in three separate studies. With the purpose of investigating aquaporin expression pattern and role in disease, different cell types representative of tissues known as players in metabolic disorders, were used as models to study specific aspects of the development of metabolic-related pathologies. The first part (subchapter 2.1), describes studies where the expression of aquaglyceroporins in human endothelial cells was detected for the first time, along with AQP1, an orthodox aquaporin with recognized impact in vascular pathophysiology. Aquaglyceroporins, although not involved in endothelial dysfunction, showed to be crucial for energy balance due to their role as glycerol channels. The second part (subchapter 2.2) gave us insights on the involvement of AQP7 and AQP12 in obesity-induced inflammation in endocrine pancreas. Using a gain/loss of function strategy, we showed the importance of AQP7 in several physiological processes such as cell proliferation, adhesion and migration. While AQP7 is involved in different inflammatory mechanisms, AQP12 seems to have a protective role in the process. This study illustrates the complexity of the settings of inflammation. The third part (subchapter 2.3) highlights the role of AQP3 in the inflammasome activation in macrophages. Taking advantage of a AQP3 inhibitor

(Auphen) and consequent blockage, this study provides evidence of AQP3 commitment in inflammation revealing a novel role of AQP3 in macrophages.

In **Chapter 3**, human blood samples were used to study AQP3 and AQP7 gene polymorphisms and relate their prevalence in a well characterized cohort of pregnant women with the development of preeclampsia and/or future hypertension. Analysis of AQP3 polymorphisms resulted in a potential tool to predict hypertension.

Chapter 4 describes the modulation of AQPs expression by dietary omega-3 fatty acids, particularly EPA and DHA. This study defines how AQP3, AQP5 and AQP7 gene expression may vary according to the adipose tissue depot location, subcutaneous and visceral white adipose tissue (WAT), and brown adipose tissue (BAT), and the influence of the n-3 long chain PUFA lipidic structures on AQPs gene expression.

Lastly, in **Chapter 5**, the results obtained along this thesis are discussed, highlighting aquaglyceroporins as potential drug targets for innovative new pharmacotherapies and presenting future considerations.

List of Abbreviations

ADA – andenosine-2',3'-dialdehyde

ADIPO – adiponectin

ALT - alanine transaminase;

Apo - Apolipoprotein

AQP(s) - Aquaporin(s)

ar/R - Aromatic/arginine

AST - aspartate transaminase

ATP - Adenosine triphosphate

BAT – Brown adipose tissue

CAT – Catalase

cAMP – Cyclic adenosine monophosphate

cDNA – Complementary deoxyribonucleic acid

CRP - C reactive protein

CVD – Cardiovascular disease

CYBA – Cytochrome b-245 alpha chain

DNA - Deoxyribonucleic acid

DHA - Docosahexaenoic acid

eNOS - Endothelial nitric oxide synthase

E-selectin – Endothelial-selectin

EPA - Eicosapentaenoic acid

FFA - Free fatty acids

G3P - Glycerol-3-phosphate

GFP - Green fluorescent protein

GK - Glycerol kinase

GLUT - Glucose transporter

HC - hip circumference
 HDL - high-density lipoprotein
 HT – Hypertensive
 ICAM-1 - Intercellular Adhesion Molecule 1
 IL - interleukin
 IR – Insulin resistance
 IRE - Insulin response element
 IRS - Insulin receptor substrate
 LDL - low-density lipoprotein
 LEP – Leptin
 LPS – Lipopolysaccharide
 MCHC - mean corpuscular hemoglobin concentration.
 MetS - Metabolic Syndrome
 mRNA – Messenger ribonucleic acid
 miRNA – Micro Ribonucleic acid
 MPO – Myeloperoxidase
 NAFLD – Non-alcoholic fatty liver disease
 NBPP – Normal blood pressure in pregnancy
 NBPPP – Normal blood pressure postpregnancy
 NOS3 – nitric oxide synthase 3
 NPA - Asparagine-Proline-Alanine
 NT – Normotensive
 PBS - Phosphate buffered saline
 PE - preeclamptic
 P_f - Water permeability coefficient (cm s^{-1})
 P_{gly} - Glycerol permeability coefficient (cm s^{-1})
 PI3K - Phosphatidylinositol-3 kinase

PLIN1 – Perilipin-1

PMA - Phorbol-12-myristate-13-acetate

PPAR α - Peroxisome proliferator-activated receptor alpha

PPAR γ - Peroxisome proliferator-activated receptor gamma

RAAS - Renin-angiotensin-aldosterone system

RDW - Red cell distribution width

SAH – S-adenosylhomocysteine

SREBP-1c, Sterol regulatory element-binding protein 1c

T2D – Type-2 diabetes

TAG –Triacylglycerol

TCA - Tricarboxylic acid cycle

TGF β - Transforming growth factor beta

TNF α – Tumor necrosis factor-alpha

VCAM-1 - Vascular adhesion Molecule 1

VLDL - Very low-density lipoproteins

VRAC – Volume-regulated anion channel

WAT – White adipose tissue

sWAT – Subcutaneous white adipose tissue

vWAT – Visceral white adipose tissue

WC - waist circumference

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Chapter I

General Introduction

This chapter is adapted from two publications in peer review journals and one book chapter:

Inês Vieira da Silva and Graça Soveral. Aquaporins in Obesity. In Aquaporins. Springer Book Series Subcellular Biochemistry, Baoxue Yang Ed., *Advances in Experimental Medicine and Biology*, Springer, Berlin, 969. (2017) DOI: 10.1007/978-94-024-1057-0_15

Inês Vieira da Silva, Joana Rodrigues, Irene Rebelo, Joana Miranda and Graça Soveral. Revisiting the Metabolic Syndrome: the emerging role of aquaglyceroporins, *Cellular and Molecular Life Sciences* (2018) DOI: 10.1007/s00018-018-2781-4

Leire Mendez-Gimenez, Silvia Ezquerro, **Inês Vieira da Silva**, Graça Soveral, Gema Fruhbeck and Amaia Rodriguez. Pancreatic aquaporin-7: a novel target for anti-diabetic drugs? *Frontiers Chemistry* (2018) DOI: 10.3389/fchem.2018.00099

Metabolic Syndrome

The International Diabetes Federation estimates that a quarter of the adults worldwide suffer from metabolic syndrome (MetS), a cluster of atherosclerotic cardiovascular disease risk factors including visceral adiposity, insulin resistance (IR) and dyslipidemia [1,2]. The main pathophysiological mechanism involved is IR (MetS is also known as IR syndrome) where liver, skeletal muscle and adipose tissue cells become progressively less sensitive to insulin and, consequently, pancreatic beta cells must secrete more insulin to maintain normoglycemia, which ultimately leads to their progressive dysfunction and eventual loss of function [3,4].

In normal physiological conditions, glucose homeostasis is tightly controlled and the fasting plasma glucose concentration is maintained within a very narrow range. In the postabsorptive state the increase in plasma glucose stimulates insulin secretion by pancreatic beta cells. Hyperglycemic challenges are tightly regulated by a normal amount of secreted insulin where hyperglycemia plus hyperinsulinemia combine to suppress hepatic glucose production and stimulate glucose uptake by splanchnic and peripheral (primarily muscle) tissues to restore normoglycemia [5]. However, under some pathological conditions glucose homeostasis may be disturbed. IR, implying depressed cellular sensitivity to insulin, is a presumed state of insufficient insulin production and insulin secretory dysfunction despite high metabolic demand. A central feature of IR is the inability to suppress free fatty acids (FFA) production and release from adipocytes by the normal levels of insulin. Indeed, adipocyte resistance to the antilipolytic effect of insulin and the consequent elevated plasma free fatty acid levels (Figure 1.1, (1)) may play an important role in the development of IR in muscle and other target tissues. Consequently, the ability of insulin to augment glucose uptake in adipose tissue, liver and muscle (Figure 1.1, (2)) is impaired. The resultant hyperglycemia (Figure 1.1, (3)) presents a stimulus to pancreatic beta cells (Figure 1.1, (4)), which being demanded to secrete large amounts of insulin as a compensatory mechanism eventually become exhausted, resulting in deregulation in the pathways of insulin action and hyperinsulinemia (Figure 1.1, (5)). In the liver, glucose production and lipid storage in the form of triacylglycerol (TAG) are increased (Figure 1.1, (6)). The enlarged TAG accumulation results in increased secretion of very-low-density lipoproteins (VLDL) into the systemic circulation and consequent dyslipidemia (Figure 1.1, (7)), an important criterion for the diagnosis of the MetS [1,6,7].

Hypertension is another symptom in MetS but its relation to the syndrome is complex, although it is almost consensual that increased blood pressure is one of the conditions in diagnostic criteria [8]. The kidney plays a crucial role in controlling blood pressure, particularly through the regulation of sodium excretion [9] and through various vasoactive systems such as the renin-angiotensin-aldosterone system (RAAS). Adipocytes also produce angiotensin-converting enzyme and cathepsins, affecting angiotensin conversion and catabolism, respectively. By increasing angiotensinogen production in adipose tissue, an enlarged fat mass may contribute to an increase in blood pressure [10]. In addition, insulin is a strong enhancer of sodium reabsorption in the kidney and, simultaneously, causes a release of nitric oxide and subsequent vasodilation. Instead, IR and concomitant hyperinsulinemia activate the RAAS and reduce nitric oxide-mediated vasodilation resulting in vasoconstriction and hypertension [11,12] (Figure 1.1, (8)). Moreover, the increase of FFA in circulation can induce angiotensinogen production and may promote oxidative stress in endothelial cells, contributing to the pathogenesis of hypertension [10].

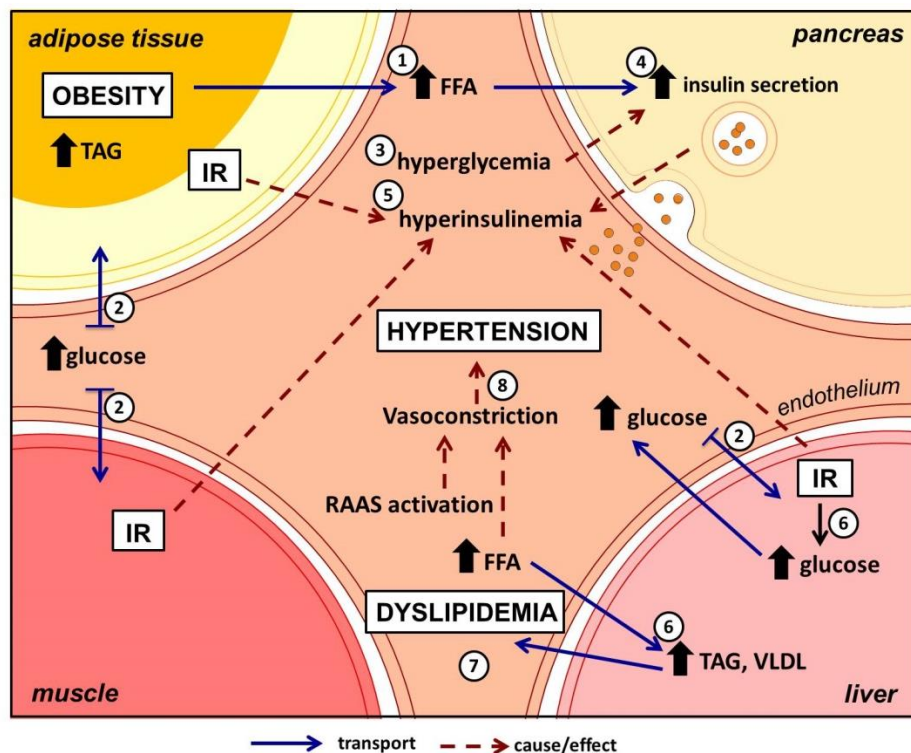


Figure 1.1. MetS results from the interplay between adipose tissue, skeletal muscle, liver and pancreas. Obesity development and adipose tissue dysfunction leads to high levels of circulating FFA (1). Decreased insulin sensitivity in liver and muscle result in impaired glucose uptake (2) and hyperglycemia (3). Over-stimulation of insulin release by endocrine pancreas (4)

ends in hyperinsulinemia (5) and consequently IR. In the liver, glucose production, lipid storage in the form of triacylglycerol and VLDL secretion are increased (6) contributing to dyslipidemia (7). Lack of insulin action combined with high levels of FFA activates the RAAS, leading to vasoconstriction and causing hypertension (8). FFA, free fatty acids; IR, insulin resistance; RAAS, renin angiotensin aldosterone system; TAG, triacylglycerols; VLDL, very low density lipoproteins.

In sum, the MetS results from the interplay between insulin-targeted tissues such as adipose tissue, liver and skeletal muscle (Figure 1.1). The involvement of these tissues in energy metabolism and metabolic dysfunction is illustrated in Figure 1.2 and will be individually addressed in the next sections.

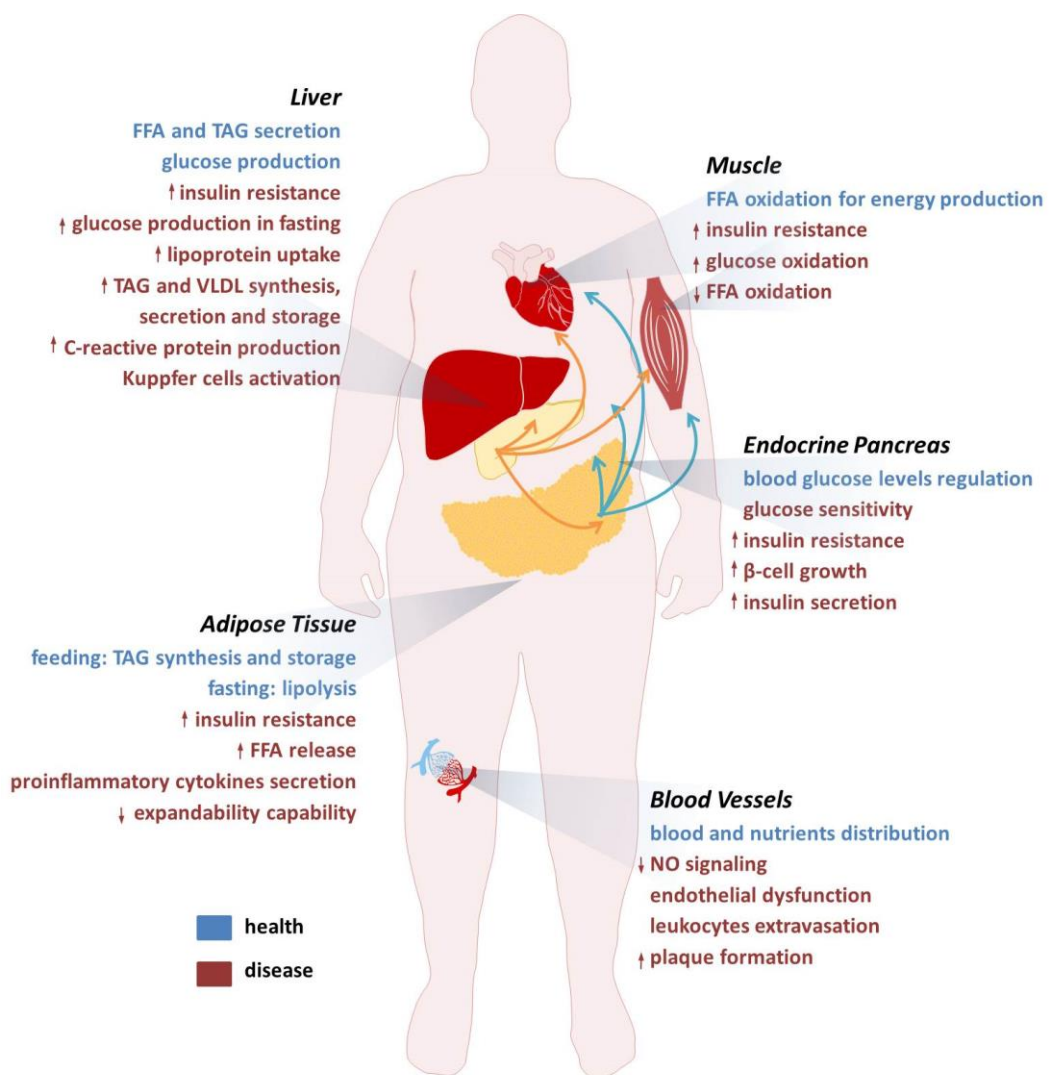


Figure 1.2. The most significant events associated with MetS. Summary of the most significant events in healthy (blue) and in disease (red) conditions affecting the main tissues implicated in MetS (adipose tissue, liver, muscle, endocrine pancreas and vessels).

Adipose tissue stores energy and regulates metabolism through the release of adipokines

Adipose tissue is a particularly flexible tissue capable of reduction, expansion or alteration under appropriate stimulations. According to its cellular and endocrine functions, the adipose tissue is divided into two major types, the white adipose tissue (WAT) and the brown adipose tissue (BAT). While white fat stores energy, brown fat expends it [13]. The current classification of adipose tissue includes a third category, the beige adipose tissue. It shows thermogenic properties similar to BAT and has been detected in humans when stimulated by cold stress or β 3-adrenoceptor agonists that mimic cold stress [14]. The three types of adipose tissues have different morphology, distribution, gene expression, and metabolic functions [15]. Being the main function of WAT the storage of energy in the form of TAG, its dysfunction is deeply implicated in obesity and related metabolic disorders.

WAT is composed by adipocytes, vascular tissue and immune cells, all enclosed in an extracellular matrix formed by proteins such as collagen. Fully mature adipocytes develop from preadipocytes after undergoing differentiation [16]. In situations of reduced energy expenditure or increased food intake, which are characterized as positive energy balance, mature adipocytes experience hyperplasia (increase in number) and hypertrophy (increase in size) to store excess lipid in the form of TAG with consequent change in their morphology [17,18,19]. Adipocyte enlargement occurs in parallel with extracellular matrix adjustment, by the action of proteases that hydrolyze collagen to allow cell hypertrophy, and with the formation of new vessels (angiogenesis) [20]. Adipocytes have origin in adipose stem cells located in the vicinity of the adipose microvasculature that, early in postnatal life or even prenatally, differentiate into preadipocytes and adipocytes by adipose vasculature stimuli [21]. Adipose tissue is an important endocrine and paracrine organ that communicates with many other organs in the body by secreting signaling proteins collectively known as adipokines [22]. In addition, adipose tissue constitutes an important source of circulating exosomal micro RNAs (miRNAs), which can regulate gene expression in distant tissues and thereby serve as a form of adipokine [23]. Overall, adipose tissue contributes to the maintenance of energy, lipid and glucose homeostasis, mediating multiple biological processes such as inflammation, immunity and metabolism [24,25,26].

Population studies have shown an association between abdominal obesity and IR. However, the specific molecular mechanisms that lead obesity to related metabolic pathologies remain unclear. Several hypotheses have been proposed to explain the development of adipose tissue dysfunction and obesity. The adipose tissue expandability hypothesis has been corroborated by both clinical and experimental data and is based on the limitation of the adipose tissue to expand above a given threshold for a specific individual [27]. When an individual increases in fat mass, the adipose tissue expands until a certain point where it reaches its limit of storage and is no longer able to accumulate more fat. At this point, ectopic accumulation of bloodstream lipids in non-adipose tissues, such as liver, skeletal muscle and pancreas occurs. Contrary to adipocytes, these tissues are poorly adjusted to store lipids, triggering a lipid-induced toxicity that culminates in failure of cellular function and results in inflammation and IR [28,29]. The maximal capacity of the adipose tissue is dependent on the type of lipid depots [25], subcutaneous or visceral, the first being more adipogenic and with greater expansion capacity (hyperplasia) and the latter metabolically more active, expanding mainly by hypertrophy with huge infiltration of macrophages and regulated by a large number of glucocorticoid receptors and a lower number of insulin receptors. It is well accepted that in humans, peripheral subcutaneous adiposity is not harmful and may even be protective, acting as lipid-buffering tissue that helps in the maintenance of the daily lipid fluxes homeostasis, whereas increased visceral fat is associated with metabolic complications due to its proximity to the liver through the portal vein together with its diminished expansion capability [26,27]. Therefore, the risk of MetS progression is closely associated with visceral obesity [30]. Moreover, evidences that the individual adipose expandability threshold is determined by genetic and environmental factors, may explain why both apparently lean and obese people are prone to develop IR [31].

Another recognized mechanism linking obesity to IR is the adipose tissue inflammation mediated by overproduction of pro-inflammatory and anti-adipogenic cytokines [32]. Hypertrophic adipocytes secrete high levels of pro-inflammatory adipokines, such as leptin, and FFA that induce macrophage infiltration into the adipose tissue and their activation. In turn, activated macrophages secrete anti-adipogenic cytokines (Tumor Necrosis Factor- α (TNF α), Interleukin-6 (IL-6) and Interleukin-1 β (IL-1 β)) that inhibit insulin action [33]. Insulin resistant adipocytes continue releasing FFA, activating macrophages to destroy compromised adipocytes that in turn secrete

even more antiadipogenic cytokines, consequently increasing IR in mature adipocytes and blocking preadipocytes maturation [27]. The cyclic mechanism of adipose tissue inflammation linking to insulin resistance is depicted in Figure 1.3.

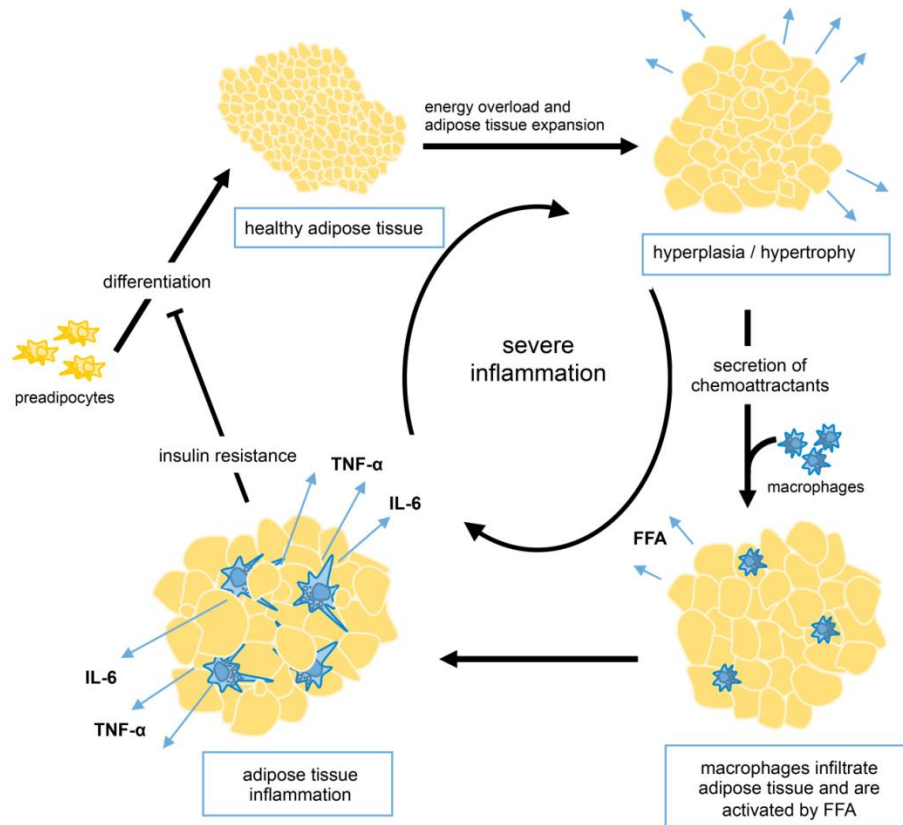


Figure 1.3. The cyclic mechanism of adipose tissue inflammation linking to insulin resistance and obesity. When the uptake of nutrients overcomes the energy expenditure, TAG accumulation in adipocytes induces adipose hyperplasia and hypertrophy, secretion of chemoattractants leading to macrophage recruitment. Large adipocytes are induced to secrete more cytokines and FFA, which in turn activate macrophages. Macrophages secrete anti-adipogenic cytokines (TNF α and IL-6) that inhibit insulin action and lead to adipose tissue inflammation. These cytokines also block the differentiation of preadipocytes into new adipocytes thus inducing the enlargement of insulin resistant-adipocytes, that continue secreting more cytokines and FFA, recruiting macrophages and leading to severe inflammation.

Liver regulates systemic energy homeostasis

The liver plays a central and critical biochemical role in metabolism. In addition to the main function of glucose uptake, production and storage as glycogen, liver is also involved in processes such as biotransformation and excretion, through bile secretion, granting a protective role from exogenous and potentially toxic substances [34]. Overall, liver acts as a buffer, regulating blood glucose levels to provide this substrate to other tissues such as brain and skeletal muscle.

In response to feeding, nutrients absorbed into the bloodstream reach the liver through the portal vein. Glucose is then converted into pyruvate, through glycolysis, in the cytoplasm of hepatocytes, being oxidized through the tricarboxylic acid cycle (TCA) and oxidative phosphorylation in the mitochondria to produce ATP. If energy is not needed, glucose is stored as glycogen, through glycogenesis, or converted into FFA and amino acids. By esterification of FFA with glycerol, TAG are generated and stored in lipid droplets or secreted into circulation as VLDL, while amino acids are used to synthesize proteins, glucose or other biomolecules [35]. These pathways are controlled by insulin, which stimulates the uptake of glucose by peripheral tissues causing a rapid removal of glucose from the blood. Insulin induces energy storage and anabolic reactions, such as fatty acid synthesis in liver and adipose tissue [36]. Moreover, insulin stimulates glycolysis and in the liver, blocks glycogenolysis and gluconeogenesis and stimulates lipogenesis [35].

During fasting, fuel substrates are released from the liver into the circulation to be metabolized in peripheral tissues. Glucagon, produced by alpha cells of the pancreas, induces an increase in hepatic intracellular cyclic adenosine monophosphate (cAMP) that promotes glycogenolysis and gluconeogenesis [37]. The net outcome of these pathways leads to an increase in hepatic glucose production.

The state of hepatic IR refers to impaired insulin suppression of gluconeogenesis. In fact, type 2 diabetics are unable to decrease glucagon appropriately upon increase in plasmatic glucose concentration [38]. Moreover, there are reports that T2D is associated with defects in insulin signaling pathway. Liver-specific insulin receptor knockout mice exhibit dramatic insulin resistance and severe glucose intolerance [39] while muscle-specific insulin receptor knockout mice display elevated fat mass, serum TAG and FFA, but blood glucose, serum insulin, and glucose tolerance are normal [40], suggesting a major role of liver in the development of hyperglycemia, T2D and MetS.

Lipid metabolism in the liver is also affected in the setting of IR. In IR condition, lipolysis in adipocytes is enhanced and an excess of FFA released from adipose tissue is delivered to the liver and muscle. In the liver, FFA are primarily used to form TAG that may accumulate causing steatosis, also known as fatty liver [8]. Due to excess FFA in the blood or inefficient FFA oxidation, TAG can also accumulate in the muscle [8]. In addition, Insulin also stimulates lipogenesis in the liver directly. There are two contrasting theories that explain the role of IR in increasing VLDL production. It has

been suggested that VLDL overproduction is a response to hyperinsulinaemia, inducing de novo hepatic synthesis of fatty acids. In fact, it was shown in obese mice that chronic hyperinsulinaemia downregulates insulin receptor substrate-2 (IRS-2), a crucial receptor in insulin signaling pathway. However, insulin continues to induce sterol regulatory element-binding protein 1c (SREBP-1c), activating fatty acid synthesis [41]. In this case, there is a combination of IR, due to increased gluconeogenesis, and insulin sensitivity, represented by elevated lipogenesis, the natural insulin response. Thus, an aggravated state of hyperinsulinaemia and consequent IR are observed. On the other hand, other study suggested that the major contributor is the increased supply of FFA to the liver, released from adipose tissue, correlating the augment in plasma FFA with the enhanced production of VLDL [42].

Skeletal muscle consumes most of the systemic glucose in an insulin dependent manner

Skeletal muscle is a key insulin-targeted tissue, constituting up to 50% of total body mass. In resting conditions it requires small amount of glucose, while 80% of blood glucose is metabolized by brain, gut and red blood cells in an insulin-independent manner [43]. However, after insulin stimulation, skeletal muscle accounts for 85% of glucose utilization [44,45,46].

Energy for muscle contraction in the form of ATP, is mostly used by myosin heads and ion pumps, especially Ca^{2+} pumps in the sarcoplasmic reticulum [47]. Insulin induces the uptake of glucose and has an antilipolytic function. In the feeding state, insulin suppresses lipolysis in the adipose tissue, declining the concentration of FFA in plasma. Under such circumstances, muscle uses mostly glucose, which uptake is stimulated, entering the cell through the glucose transporter GLUT4, whose translocation from intracellular vesicles to the plasma membrane is induced by insulin [48].

On the other hand, under fasting conditions, muscle uses mostly FFA for energy production, as these are in high concentrations in plasma due to adipose tissue lipolysis, increasing muscle fatty acid oxidation [49]. High-fat diets play a role in the development of IR in muscle. In fact, a study performed in rats showed that an increased plasma concentration of fatty acids leads to an increased intracellular concentration of fatty acyl-coA which in turn blocks insulin activation of insulin receptor substrate-1 (IRS-1) [50]. As a final result, this will cause a decrease in insulin

sensitivity. IR is characterized by a decreased but also delayed muscle glucose uptake [51]. Moreover, it was reported that in IR states such as obesity, the rate of FFA oxidation is reduced during fasting despite similar plasmatic concentrations [52]. Impaired fat oxidation can be associated to mitochondrial defects since it occurs in this organelle. In fact, patients with controlled T2D show reduced muscular ATP synthesis [53] and decreased activity of several mitochondrial enzymes [54], reflecting problems in oxidative phosphorylation. The increased FFA influx and decreased mitochondrial fat oxidation occurring in IR alters the dynamic equilibrium between fatty acid intake and oxidation and may be reflected in elevated muscular lipid content.

Aquaporins: new players in the development of MetS

Aquaporins (AQPs) are a family of membrane protein channels, present in all kinds of organisms [55] that facilitate the transport of water and small molecules such as glycerol and urea through the plasma membrane driven by osmotic or solute gradients [56,57]. The members of the aquaporin family present high homology in protein sequence [58] and so far, 13 isoforms (AQP0-12) were described in mammals with tissue- and subcellular-specific localization [59] suggesting a link between site of expression and function [60]. According to their primary sequence and channel selectivity, AQPs are divided into three subfamilies: orthodox AQPs (AQP0, 1, 2, 4, 5, 6, 8) are mainly selective for water, aquaglyceroporins (AQP3, 7, 9, 10) facilitate the permeation of other small-uncharged solutes such as glycerol in addition to water, and S-aquaporins (AQP11, 12) found mostly intracellularly, with lower sequence homology and permeability still unclear [61].

AQPs are architected in membranes as tetramers formed by four identical monomers (Figure 1.4A and B), each behaving as a channel and sharing a conserved typical hourglass fold (Figure 1.4D). Each monomer is composed by around 320 amino acid residues with approximately 28 kDa, characterized by a topology of six highly hydrophobic transmembrane domains (1–6) connected by five loops (A–E) with the N and C-terminal sequences facing the cytosol, and two half-helices containing highly conserved asparagine–proline–alanine (NPA) motifs that are part of the protein family signature [55] (Figure 1.4C). In order to achieve the channel selectivity, the pore region contains two selective filters with size constrictions and charge characteristics that enable water or glycerol to permeate, while preventing the passage of charged

molecules like protons or any solute above the pore size [62]. Starting from the periplasmic side of the channel, the first selectivity filter is a constriction site formed by aromatic/arginine residues (ar/R) near the extracellular entrance [63] that determines the size of molecules allowed to pass through (pore size of ~ 2.8 Å in orthodox aquaporins and $\sim 3,4$ Å, in aquaglyceroporins). The second constriction site is formed by the two highly preserved NPA motifs that behave as dipoles, preventing ions to permeate the channel [64,65] (Figure 1.4D).

AQPs have been associated with a variety of important physiological roles including transepithelial fluid transport, brain water homeostasis, osmoregulation, cell migration and proliferation [57,66] and have been suggested as potential targets for drug development [67,68,69,70]. In particular, due to their ability to transport glycerol through the plasma membrane regulating cellular glycerol content, aquaglyceroporins are involved in skin hydration [71] and fat metabolism [72] and are emerging as important players in adipose tissue homeostasis and insulin response with possible implications in metabolic disorders such as obesity and MetS [68,69].

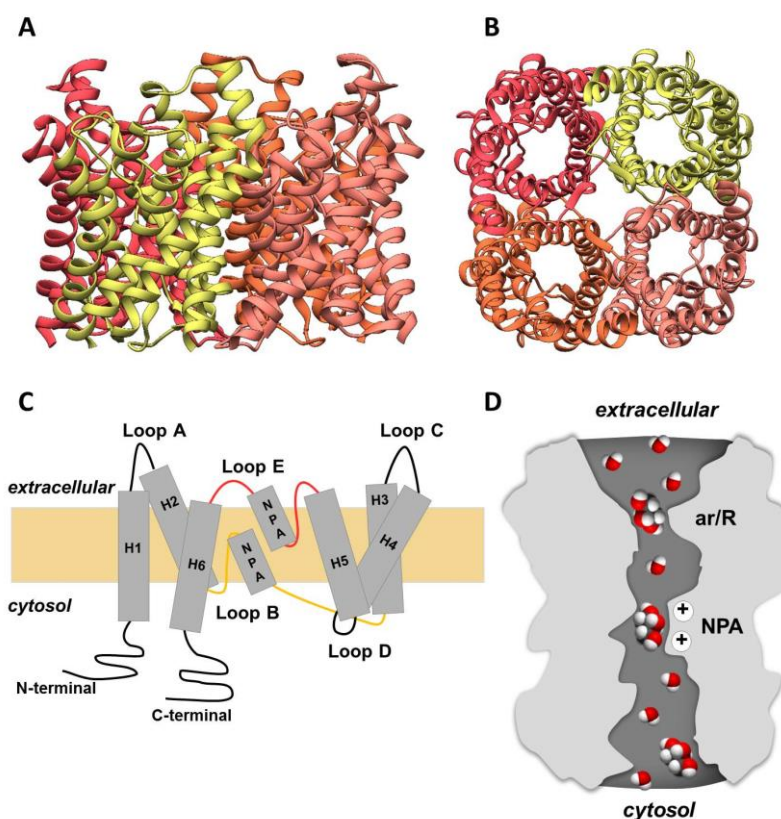


Figure 1.4. General structure of aquaporins. (A) Intracellular and (B) side views of the homotetrameric representation of the glycerol channel GlpF based on its X-Ray structure (PDB code: 1FX8). Figures were generated with UCSF Chimera software. (C) Representation of AQP membrane topography, showing the monomer comprising six membrane-spanning α -helices (H1-6) connected by five loops (A-E), the conserved asparagine–proline–alanine (NPA)

motifs embed in the membrane. In the functional monomer, the hydrophilic loops B and E are bent back into the cavity formed by the helices. The two loops meet in the middle to form the water-selective gate that contains two consensus NPA motifs (Asn-Pro-Ala). The hydrogen bonding properties of the polar side groups of the two Asn residues are thought to constitute the permeation barrier. **(D)** Illustration of water molecules permeating the aquaporin pore, with the two selectivity filters ar/R and NPA represented.

Aquaglyceroporins in glycerol metabolism and energy homeostasis

The ability of aquaglyceroporins to facilitate glycerol permeation through membranes and its impact in metabolic disorders has raised attention to the involvement of glycerol in metabolism and in a variety of pathophysiological mechanisms.

Glycerol (1,2,3-propanetriol) is a polyalcohol that can be produced intracellularly from various metabolic sources such as glucose, protein and glycerolipid metabolic pathways (endogenous glycerol) as well as taken up from dietary fats released during digestion (exogenous glycerol). Glycerol is the carbon backbone for the de novo synthesis of TAG and phospholipids and it is also an important intermediate in both carbohydrate and lipid metabolism. Intracellular glycerol accumulation activates glycerol kinase (GK) promoting the synthesis of glycerol-3-phosphate (G3P), a by-product from glycolysis and a key molecule in the regeneration of NAD⁺ from NADH [73,74]. In the liver, G3P is used in glycolysis and gluconeogenesis, and, in muscle it is an energy substrate via the G3P shuttle, which has a key role in oxidizing glucose rapidly and generating adenosine triphosphate (ATP) in the mitochondria through the oxidation of G3P [75]. Under negative energy balance conditions (fasting or exercise), TAG stored in WAT are hydrolyzed releasing FFA and glycerol into the bloodstream, that can be used by other tissues as an energy source [76,77]. Being a highly hydrophilic molecule, glycerol diffusion across tissues' membranes is facilitated through protein channels that allow its rapid transfer and promote its availability for intracellular metabolism. In this regard, the members of the aquaglyceroporin sub-family have been recognized as the main membrane channels involved in glycerol permeation. Regulation of glycerol fluxes via aquaglyceroporins plays a key role in the control of metabolic processes and energy homeostasis [74,78,79]. A list of the aquaglyceroporins expressed in organs and tissues involved in energy homeostasis and their implication in glycerol fluxes is presented in Table 1.1.

Table 1.1. Expression of aquaporins by organ/tissue and their implication in glycerol balance.

Organ/Tissue	Aquaglyceroporin	Glycerol balance
Small Intestine	AQP3 [80]	Glycerol secretion and enterocyte proliferation [81]
	AQP7 [82]	Rapid glycerol absorption in villus epithelium [82,83]
	AQP10 [84,85,86]	Carrier and channel for glycerol and other solutes transport [83]
Adipose Tissue	AQP3 [74]	Glycerol metabolism [74]
	AQP7 [87,88,89]	Main glycerol transporter; control glycerol uptake and release [72,89,90]
	AQP9 [74]	Glycerol influx [74]
	AQP10 [89]	Maintain normal glycerol levels [89]
	AQP11 [91]	Mediate intercellular glycerol movements [91] ???
Cardiac and Skeletal Muscle	AQP3 [75,92,93]	Glycerol transport for energy production in skeletal muscle [92]
	AQP7 [75,92,93]	Glycerol transport for energy production mainly in cardiac muscle [75,92,94]
Liver	AQP9 [95]	Uptake of glycerol for glucose production [95]
Endocrine Pancreas	AQP7 [96]	Regulation of insulin production/secretion [96,97]
Kidney	AQP7 [98]	Involved in glycerol reabsorption [99,100,101]

Figure 1.5 depicts a schematic model showing the involvement of aquaglyceroporins in glycerol metabolism and energy balance. The expression and localization of the various aquaglyceroporins in multiple organs is important to assure glycerol fluxes across tissues and is crucial for glycerol metabolism and homeostasis. A detailed description can be found in Figure 1.5 legend.

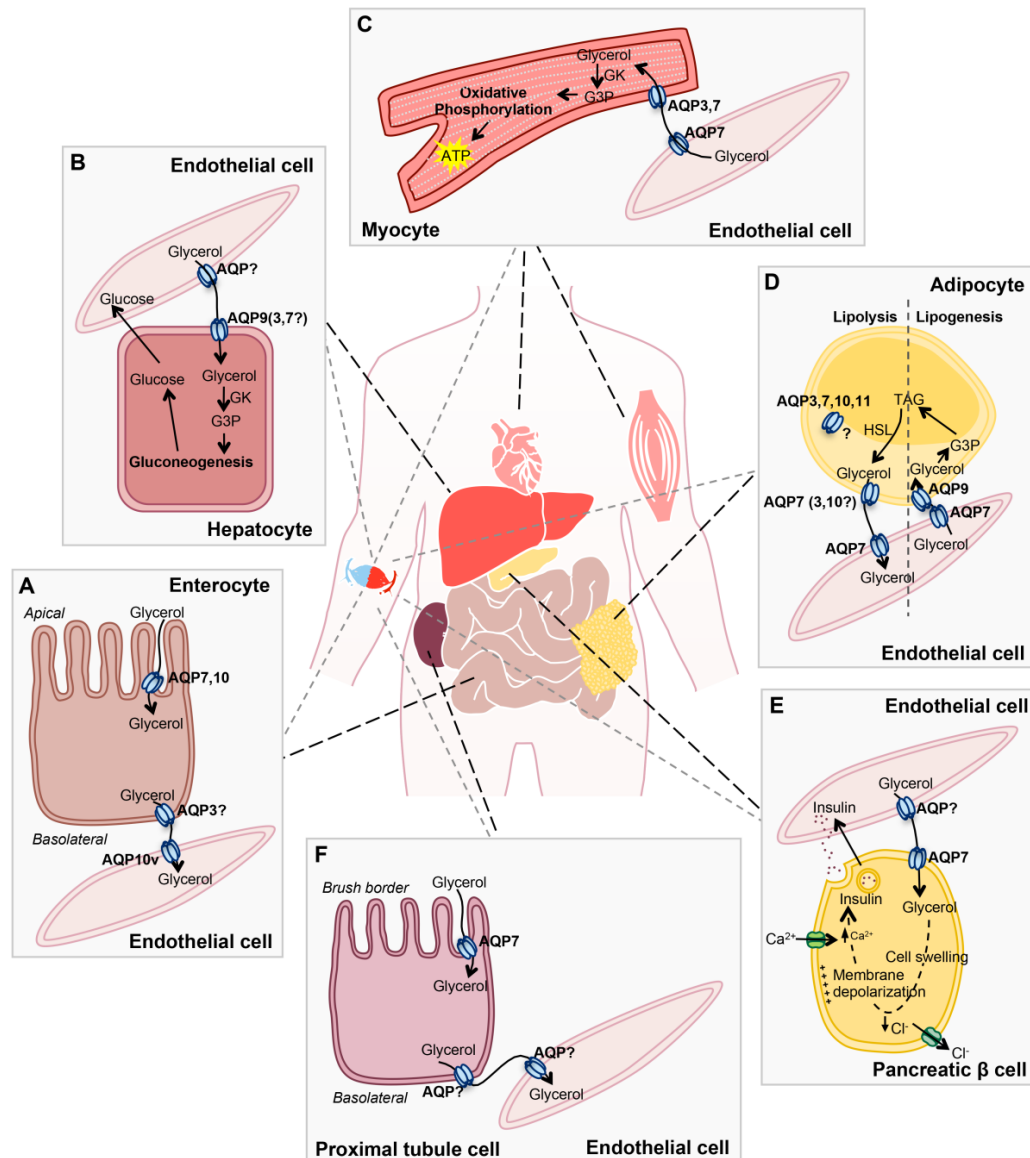


Figure 1.5. Involvement of aquaglyceroporins in glycerol metabolism and energy homeostasis. (A) Glycerol from dietary fat is absorbed via AQP7 and AQP10 in the apical membrane of small intestine epithelial cells and exits via AQP3 located in the basolateral membrane. Endothelial cells from adipose vasculature express AQP10 variant that allows glycerol entry to the bloodstream to be used by other organs as energy source. (B) Glycerol is taken up to the liver by AQP9 (and possibly also AQP3 and AQP7) expressed at the basolateral sinusoidal membrane of hepatocytes. In the hepatocyte, glycerol is converted to G3P by GK to be used in gluconeogenesis. (C) Glycerol is used in cardiac and skeletal muscle as an energy source. It permeates the membrane by AQP3 and 7 and, after conversion in G3P by GK, is used for ATP production. (D) In fasting conditions when lipolysis occur in the adipocytes, TAG hydrolysis yields FFA and glycerol that is released to the blood via AQP7 (and possibly also 3 and 10?) expressed in both adipocyte and endothelial cell membranes. In feeding conditions when plasma glycerol reaches high concentrations, glycerol is taken up by adipocytes possibly via AQP9 being converted to TAG and stored in the lipid droplets. (E) In response to high plasma nutrient levels, glycerol enters pancreatic β cells via AQP7 and participates in a cascade of events that culminates with insulin exocytosis. (F) Glycerol filtered in the kidney is reabsorbed to the blood via AQP7 expressed in the brush border membrane of proximal tubule cells, thus preventing its excretion in the urine.

AQP7 is the main glycerol gateway in adipocytes

Adipose tissue constitutes the most important source of glycerol in the body. AQP7 is the most representative glycerol channel and the first identified in human and rodents adipose tissue [98,102,103], adipocytes [87,88] and adipose endothelial cells [89,99,101,104]. Nevertheless, alternative glycerol pathways have also been detected in human adipocytes, such as AQP3 [74,88,105], AQP9 [106], AQP10 [89] and AQP11 [91].

The fundamental role of AQP7 in facilitating glycerol efflux from the adipocyte into the bloodstream was achieved after its characterization as a glycerol channel [87]. Obese insulin-resistant db+/db+ mice showed higher AQP7 expression [87] and similarly, an increase in AQP7 mRNA was also observed in adipose tissue of a rodent model of T2D with obesity [107]. This suggests that AQP7 dysregulation may lead to an augmented input of glycerol for hepatic gluconeogenesis and, consequently, to increased glucose in the bloodstream, in T2D [98]. Furthermore, AQP7 knockout mice showed adipocyte hypertrophy and early obesity onset due to the accumulation of glycerol, which stimulates GK, leading to TAG accumulation [72,90]. Controversially, susceptibility for obesity was not observed in other AQP7 knockout mice [99,108]. Despite the different phenotypes described in distinct AQP7 knockout mice, all confirmed the involvement of AQP7 in glycerol metabolism. Moreover, although no obvious correlation was found between adipose AQP7 expression/glycerol metabolism and related metabolic complications in humans [109], genome-wide analysis found AQP7 gene linked to T2D [110], MetS [111] and obesity, the latter just for female participants [112].

Gender differences concerning the role of AQP7 in adipose tissue metabolism have also been reported, where higher fasting circulating levels of glycerol have been found in women comparing to men. Obese women showed higher AQP7 expression than men in both subcutaneous and visceral adipose tissue, suggesting an association between impaired AQP7 expression and obesity, at least in women [104,113]. AQP7 gene expression is upregulated in mice and human in the fasting state or during exercise, whereas during the feeding state it is downregulated and its abundance is inversely related with plasma insulin levels [87]. Transcription of AQP7 gene is inhibited by the increase of plasma insulin levels through a negative insulin response element (IRE) located in the promoter region of AQP7 gene [114,115] and by blockage of the

phosphatidylinositol-3 kinase (PI3K) pathway [106,114]. On the other hand, peroxisome proliferator-activated receptor gamma (PPAR γ) was demonstrated to upregulate AQP7 [79,109,114]. PPAR γ is also essential for adipocyte differentiation by regulating the transcription of several adipose genes, including AQP7, and, in differentiating adipocytes, an increase in AQP7 expression along with adipocyte differentiation was observed, suggesting a cell differentiation-dependent regulation [87,116]. Indeed, thiazolidinediones (synthetic PPAR γ) and insulin sensitizers were reported to upregulate AQP7 [107,114,115], whereas insulin resistance inducers such as leptin [106,113], TNF α , adrenergic agonists and steroids, downregulate AQP7 expression [117]. Lipogenic hormones, such as ghrelin, also control AQP7 regulation, decreasing its expression while promoting TAG accumulation [118]. During fasting, low plasma insulin levels and catecholamine induce AQP7 gene transcription and AQP7 translocation to the plasma membrane, promoting glycerol release from adipocytes [87].

A recent study in human primary adipocytes proposes a molecular mechanism explaining how glycerol release is controlled in adipocytes by interaction of AQP7 with perilipin 1 (PLIN1) and protein kinase A (PKA). In lipogenic conditions, AQP7 and PLIN1 physically interact in the adipocyte whereas in the lipolytic state, catecholamine-activated PKA phosphorylation of the N-terminus of AQP7 reduces the complexation favoring AQP7 translocation to the plasma membrane [119]. Studies with obese individuals showed a differential regulation of AQP7 depending on the type of adipose depot, subcutaneous or visceral; low AQP7 expression was detected in subcutaneous fat leading to fat accumulation and adipocyte hypertrophy. In contrast, increased AQP7 levels were observed in visceral fat, which can be correlated with increased lipolysis [74,79]. Knowing that subcutaneous adipose tissue is more insulin sensitive than visceral adipose tissue, AQP7 downregulation might represent a mechanism of protection attempting to prevent lipid depletion and consequent lipotoxicity to peripheral organs [79].

AQP9 facilitates glycerol uptake in the liver for gluconeogenesis and TAG synthesis

Glycerol has a central role in the equilibrium of hepatic gluconeogenesis and lipid accumulation in adipose tissue. In the liver, AQP9 is the channel responsible for glycerol influx both in the fed or fasting states [74,95,120] where it is converted to G3P

used for gluconeogenesis and synthesis of TAG [106]. In addition to AQP9 expressed in the basolateral sinusoidal membrane and AQP11 in the endoplasmic reticulum, aquaglyceroporins AQP3 and AQP7 were also found, but their exact location and physiological roles are still unclear [74,106,121,122]

The role of AQP9 as the major glycerol channel in the liver and its implication in glycerol metabolism was elucidated through experiments with AQP9-knockout mice [95] that showed increased plasma glycerol and TAG due to impaired glycerol metabolism. In healthy conditions, during the feeding state, insulin leads to a reduction in lipolysis and AQP7 downregulation that results in impaired glycerol release from adipose tissue, and parallel liver AQP9 downregulation and impaired glycerol uptake, a coordinated regulation mechanism able to prevent liver gluconeogenesis [123,124].

Liver AQP9 appears to be downregulated only in male rodents whereas in female, downregulation is prevented by estrogen [125]. Gender-specific differences in glycerol metabolism were also reported in healthy humans probably due to hormonal regulation of adipose and liver aquaglyceroporins, which may impact on the relative risk of metabolic diseases in men and women [113].

The role of AQP9 in fat accumulation liver disorders was investigated [124,126,127,128]. Using a non-alcoholic fatty liver disease (NAFLD) cell model, expression of AQP9 led to steatosis due to increase of intracellular TAG, FFA and glycerol contents and importantly, steatosis was reversed by AQP9 suppression [128]. Although correlation between hepatic AQP9 expression and steatosis in obese patients could not be found [129], downregulation of hepatic AQP9 along with NAFLD or non-alcoholic steatohepatitis was reported in obese patients with IR or diabetes, suggesting a compensatory mechanism to avoid TAG, FFAs and glycerol accumulation and gluconeogenesis [127]. Further investigation is needed to clarify the role of AQP9 in liver metabolic homeostasis and fat liver disease.

AQP3 and AQP7 transport glycerol for energy production in muscle

In mammalian cardiac and skeletal muscle aquaglyceroporins facilitate glycerol uptake for energy production [75,94]. As an energetic substrate, glycerol is dispensable under physiological conditions but becomes important in the stressed heart [75]. Expression of AQP1, 3, 4 and 7 was found in cardiac muscle [75,92,93], where AQP1

and 4 are involved in water permeation and AQP3 and 7 have a role in glycerol transport particularly for energy production [92]. While AQP3 mRNA was identified in human and rat heart tissues, AQP3 protein was weakly expressed or absent in human cardiomyocytes [75,80,93].

So far, AQP7 is the only aquaglyceroporin known to be involved in glycerol permeation in mammalian heart muscle, confined to myocytes and fibroblasts [75,93,99,130]. AQP7-knockout mice showed lower glycerol and ATP contents in cardiomyocytes and cardiac muscle along with lower glycerol uptake and, in pressure overload condition, developed excessive cardiac hypertrophy with higher mortality [75]. These results supported AQP7 involvement in cardiac muscle glycerol uptake as well as glycerol relevance as a source for cardiac energy [75,94].

The preferred metabolic substrate for heart muscle energy in diabetes mellitus is glucose instead of FFA and glycerol, due to activation of the genes responsible for glucose oxidation and downregulation of genes related with FFA oxidation [131,132]. The increased levels of blood glycerol and its continuous removal, mainly via AQP7, as well as the fact that it is not being used for oxidative phosphorylation, may explain the high TAG accumulation and storage inside myocytes. A few AQPs were identified in mammalian skeletal muscle. AQP3 was first described in rat skeletal muscle [133] and its expression was confirmed in human [80,134]. Along with AQP4, AQP3 was suggested to participate in the maintenance of water homeostasis in myofibers of skeletal tissue [135]. AQP9 was found weakly expressed in mice skeletal muscle but its role has not been defined [136]. AQP7 was found expressed in human and mice skeletal muscle [134,137], however a later study described its location in capillaries instead of in sarcolemma of skeletal muscle fibers [99]. Although the functional role of AQP7 in human skeletal muscle is still not clear, the fact that insulin decreases its expression in mice adipose tissue in parallel with TAG content in muscle [138] supports AQP7 glycerol channeling as a mechanism involved in skeletal muscle metabolic processes.

AQP7 is implicated in the signaling cascade for insulin exocytosis

Pancreatic β -cells are responsible for synthesizing, storing and secreting insulin in response to an increase in blood nutrients such as glucose, amino acids and FFAs. It was demonstrated that a raise in blood glucose induces pancreatic β -cell swelling,

activates volume-regulated anion channels (VRAC) [139]. The subsequent chloride efflux and cell membrane depolarization opens voltage-sensitive calcium channels allowing calcium influx and triggering a signalization cascade that results in insulin secretion [96,97,140,141]. Due to the basal low intracellular GK concentration, glycerol is not likely to directly induce insulin secretion. However, similar to the effect of glucose, pancreatic islets confronted to an isosmotic or hyperosmotic solution of glycerol undergo cell swelling, activation of VRAC and depolarization with subsequent insulin release [96]. The putative role of glycerol and aquaporin expression in regulation of insulin secretion was intensively studied. AQP7 is the main glycerol channel in the endocrine pancreas and mediates the rapid entry of extracellular glycerol into β -cells [108,142]. Insulin secretion was impaired in pancreatic islets from AQP7 knockout mice by the addition of glycerol or increasing D-glucose concentrations. Exposure of β -cells to hypotonic or hypertonic medium caused a similar degree of swelling and comparable pattern of electrical activity in cells from wild type and AQP7 knockout mice. However, when glycerol was added to the extracellular medium these events were only reduced in AQP7 knockout cells [97]. On the other hand, AQP7 knockout mice exhibit increased intra-islet glycerol and TG content with a concomitant increase of GK activity, increased pancreatic insulin mRNA levels and hyperinsulinemia [108], suggesting that AQP7 reduction favors intracellular glycerol accumulation for TAG biosynthesis, insulin biosynthesis and secretion and may play an important role in the exocytosis pathway of insulin [140]. Recently, AQP12 was also found expressed in β -cells of the Langerhans islets, both in samples of rat pancreas and in RIN-m5F β -cells, a widely used cell line based on its high insulin secretion rate [143]. However, AQP12 expression was not correlated with TAG accumulation or with insulin release [143].

Endothelial aquaporins: the gateway between bloodstream and adjacent tissues

The endothelium, a confluent layer of endothelial cells, is the biological gateway between the blood and organs and is the source of many factors that are critical to health and disease. In particular, glycerol obtained from dietary fat or from adipocyte TAG hydrolysis must cross endothelial membranes to reach the tissues where it will be processed. It has been proposed that aquaglyceroporins expressed in endothelia might be involved in glycerol transmembrane flow. AQP7 was found in mice capillary

endothelia of adipose tissue (and cardiac and striated muscle) and was upregulated in streptozotocin-induced diabetes mellitus [99] suggesting that insulin effect on adipose tissue also involves the control of glycerol efflux out of endothelial microvessels. Accordingly, human AQP7 was found localized at the plasma membrane of capillary endothelial cells and adjacent adipocytes [105], and may represent the main route for endothelial transmembrane glycerol fluxes. However, our recent study in human endothelial primary cells (HUVEC) revealed AQP3 as the predominant aquaglyceroporin accounting for glycerol fluxes in these cells, suggesting that AQP3 is also an important glycerol gateway between bloodstream and adjacent tissues [144].

Impaired glycerol fluxes in the onset of MetS

The involvement of aquaglyceroporins in adipose tissue dysfunction and consequent lipotoxicity-induced complications has been in debate in the last years. Putative mechanisms for the role of aquaglyceroporins in the onset of the metabolic syndrome include their altered expression and coordinated regulation in the tissues involved, such as adipose tissue, liver and muscle. A possible mechanism in which impaired glycerol fluxes and metabolism are key factors to the development of MetS is illustrated in Figure 1.6. In the adipose tissue, during fasting glucagon promotes lipolysis with glycerol release into the bloodstream via AQP7, 3 and 10, and in response to feeding insulin promotes lipogenesis with storage of glycerol as TAG. Balance of lipolysis and lipogenesis is crucial to maintain a healthy adipose reservoir. However, in case of high food intake and sedentary lifestyle when body energetic demands are largely overcome, adipose tissue TAG storage reaches its limit of expandability and lipotoxicity occurs. Overproduction of pro-inflammatory adipokines such as $\text{TNF}\alpha$, IL-6, IL-1 β and leptin, and reduced production of anti-inflammatory and insulin sensitizing adipokines lead to adipose tissue dysfunction [24,145], affecting aquaglyceroporin regulation differently in subcutaneous and visceral fat. In subcutaneous adipose tissue, AQP7 downregulation and impairment of glycerol release leads to fat accumulation. In visceral adipose tissue, AQP7 and AQP3 are upregulated allowing increased effluxes of glycerol and FFA, hormones and proinflammatory cytokines to the portal vein to be delivered to the liver, where they interact with hepatocytes and various immune cells such as Kupffer cells [146]. In the liver, excessive glycerol levels imported via AQP9 will be used for de novo lipogenesis in

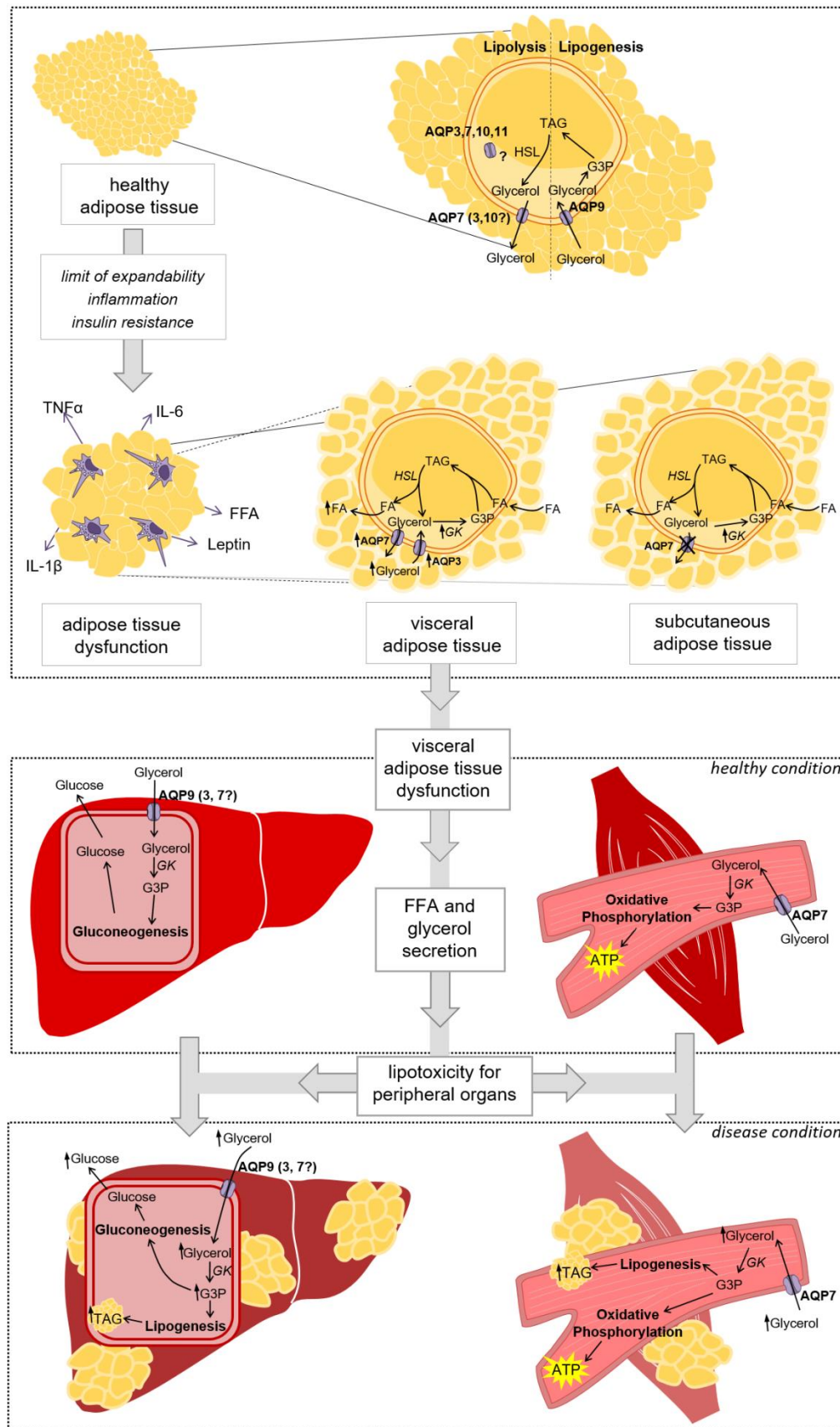


Figure 1.6 - Involvement of aquaglyceroporins in adipose tissue dysfunction and lipotoxicity in liver and muscle. (A) In healthy conditions, during fasting when blood nutrient levels are low, TAG hydrolysis in the adipocytes yields FFA and glycerol that is released to the

blood stream mainly via AQP7. In the feeding state, when plasma glycerol reaches high concentrations, glycerol is taken up by adipocytes possibly via AQP9, and is converted to TAG that is stored in lipid droplets. When adipose tissue expandability reaches its limits, macrophages infiltration and activation by secretion of proinflammatory molecules (TNF α , IL-1 β , IL-6, FFA, leptin) prompt inflammation, loss of insulin sensitivity and adipose tissue dysfunction. In disease, AQP7 downregulation in the subcutaneous adipose tissue results in increase in intracellular glycerol supply for TAG synthesis, which accumulates in lipid droplets with consequent adipocyte hypertrophy. When adipocytes reach their limit of fat accumulation, upregulation of AQP3 and AQP7 in the visceral adipose tissue facilitates the efflux of glycerol into the bloodstream that together with high levels of FFA trigger lipotoxicity in peripheral organs. **(B)** In healthy conditions, blood glycerol is taken up by AQP9 in the liver (and possibly by AQP3 and 7) expressed in the basolateral sinusoidal membrane of hepatocytes, and is converted in G3P to be used in gluconeogenesis. In cardiac and skeletal muscle, glycerol is taken up by AQP7 and it is used to generate reductive power in oxidative phosphorylation for ATP production. **(C)** In disease, higher blood glycerol levels stimulate a rise in intracellular G3P in the liver and muscle that can be used for de novo lipogenesis with resultant intracellular TAG accumulation and lipotoxicity. FFA, free fatty acids; G3P, glycerol-3-phosphate; GK, glycerol kinase; TAG, triacylglycerols.

addition to gluconeogenesis. High glycerol in the blood has also a lipotoxic effect in muscle. In the myocyte, glycerol imported via AQP7 is an energy source through oxidative phosphorylation but in case glycerol is increased in the blood, it is also used for TAG synthesis. Accumulation of TAG in liver and muscle, which are not appropriate fat storage depots, leads to cell dysfunction and failure of their tissue-specific functions.

Aquaporins as drug targets for obesity and metabolic-related complications

The implication of aquaglyceroporins in fat metabolism and obesity indicates that from a pathophysiological point of view these proteins are promising drug targets. The possibility of regulating the expression of aquaglyceroporins, in particular AQP7 in adipose tissue, offers a potential therapeutic approach for the regulation of fat accumulation and treatment of obesity. Altered AQP7 expression by hormones (insulin, catecholamines), cytokines and adipokines [106,117,118,124] is described in the previous section. As for AQP7 channel activity, the only specific modulator described so far is the gold compound Auphen that showed to inhibit glycerol permeability in an adipocyte cell line [91]. However, while Auphen may have a possible application in diagnosis to uncover AQP7 activity and gating [147,148], strategies to treat obesity point towards activation of AQP7 function rather than inhibition. Thus, the design of small-molecule upregulators of AQP7 expression and function is of utmost interest and would undoubtedly have therapeutic applications. A recent study reported that

supplementation of high-fat diets with apple polyphenols impaired adipocyte hypertrophy and prevented adiposity increase by a mechanism that included increased AQP7 and leptin mRNA levels in rat visceral adipocytes [149]. However promising, the complex mixture of polyphenols included in this functional food may hinder the identification of structure-activity relationships of the phenolic substances. Hence, further investigations to untangle aquaporins interplay with other adipose regulatory molecules, regulation by hormones and possibility of channel gating, are needed to better establish the mechanistic basis of AQPs involvement in pathogenesis of obesity, which is crucial for the identification of novel modulators design of new compounds.

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Chapter II

Aquaporins in Obesity and Related Metabolic Complications

2.1. Endothelial aquaporins and hypomethylation: potential implications for atherosclerosis and cardiovascular disease

This subchapter is adapted from one publication in a peer review journal:

Inês Vieira da Silva[#], Madalena Barroso[#], Teresa Moura, Rita Castro and Graça Soveral. Endothelial Aquaporins and Hypomethylation: Potential Implications for Atherosclerosis and Cardiovascular Disease. *International Journal of Molecular Sciences*. 19 (2018). (DOI: 10.3390/ijms19010130)

[#] Equal contribution

Abstract

Aquaporins (AQPs) are transmembrane channels that facilitate water and glycerol permeation through cell membranes. Recently, the water channel AQP1 was suggested to contribute to endothelial homeostasis and cardiovascular health. Less is known about endothelial aquaglyceroporins expression and its implication in cardiovascular disease (CVD). We have previously used cultured human endothelial cells under a hypomethylating environment to study endothelial dysfunction and activation, a phenotype implicated in the establishment of atherosclerosis and CVD. Here, we used the same cell model to investigate aquaporin's expression and function in healthy or pro-atherogenic phenotype. We first confirmed key features of endothelium dysfunction and activation in our cell model, including an augmented endothelial transmigration under hypomethylation. Subsequently, we found AQP1 and AQP3 as the most predominant AQPs accounting for water and glycerol fluxes, respectively, in the healthy endothelium. Moreover, endothelial hypomethylation led to decreased levels of AQP1 and impaired water permeability without affecting AQP3 and glycerol permeability. Furthermore, TNF α treatment induced AQP1 downregulation suggesting that the inflammatory NF- κ B signaling pathway mediates AQP1 transcriptional repression in a pro-atherogenic endothelium, a possibility that warrants further investigation. In conclusion, our results add further support to AQP1 as a candidate player in the setting of endothelial dysfunction and CVD.

Keywords: aquaporins; hypomethylation; S-adenosylhomocysteine; water and glycerol permeability; endothelial dysfunction

Introduction

Aquaporins (AQPs) are a family of highly conserved transmembrane protein channels [1] that facilitate the transport of water and small non-charged molecules (such as glycerol) across cell membranes [2,3]. In humans 13 isoforms (AQP0-12) were described with high amino acid sequence homology and tissue- and subcellular-specific localization [4,5] suggesting a link between the site of expression and physiological relevance [6].

AQPs are grouped based on their primary sequences and permeation selectivity. Orthodox AQPs are considered strict water channels (AQP0, 1, 2, 4, 5, 6, 8, although AQP6 and AQP8 are also involved in the transport of anions [7] ammonia [8]), whereas aquaglyceroporins facilitate the movement of glycerol and other small solutes in addition to water (AQP3, 7, 9, 10); unorthodox AQPs are found mostly intracellularly, with lower sequence homology and permeability not defined (AQP11, 12) [9,10].

Consistent with their wide tissue distribution, AQPs are associated with diverse physiological and pathophysiological processes [11]. Among the orthodox AQPs, AQP1 was found as the major water channel expressed in endothelial membranes supporting a major role in transcellular water movement throughout the body [12,13]. Notably, the importance of AQP1 to maintain endothelial homeostasis and cardiovascular health has been recently reported [14,15]. Endothelial dysfunction is a well-established response to cardiovascular risk factors that precedes atherosclerosis development. It was documented that AQP1 is expressed in human atherosclerotic lesions and its deficiency was related to endothelial dysfunction and atherosclerosis progression [15]. In another example illustrating the importance of AQPs in health, besides a strong involvement in gastrointestinal pathophysiology [16], aquaglyceroporins are emerging as key players in adipose tissue homeostasis and insulin response with potential implications in obesity and metabolic-related complications, such as metabolic syndrome and cardiovascular disease (CVD) [17]. Within the aquaglyceroporin subfamily, AQP7 and AQP9 are the most abundant glycerol channels in adipose tissue and liver respectively, and their coordinated regulation was shown to have a crucial role in maintaining body energy homeostasis [18,19]. During fasting, glycerol produced from triglyceride lipolysis in adipocytes is channeled by AQP7 into the bloodstream and is taken up by AQP9 in the liver for gluconeogenesis [20]. Knowing that the endothelium is a major regulator of tissue homeostasis and metabolite fluxes, endothelial glycerol channels may have a crucial role in tissue glycerol entry/exit through vascular endothelium. In fact, by immunohistochemistry analysis AQP7 expression was detected in capillaries of adipose tissue, cardiac and striated muscle [21,22] and an AQP10-variant expressed in the capillary endothelium in villi of the small intestine [23], suggesting an active involvement in glycerol absorption and distribution to tissues. AQP9 was also identified in pial vessels in the brain, where its main role was related to water transport through the blood-brain barrier [24]. However, besides capillary tissue expression,

aquaglyceroporin subcellular transport properties in endothelia have not been investigated.

Increased concentrations of plasma homocysteine, or hyperhomocysteinemia negatively impact endothelial homeostasis and have been established as a common risk factor for CVD by mechanisms incompletely defined [25,26]. A current view holds that S-adenosylhomocysteine (SAH), the homocysteine precursor that accumulates in the setting of hyperhomocysteinemia, is the important mediator of homocysteine-associated atherosclerosis and CVD [27,28,29]. Supporting this possibility, our previous results clearly present SAH as a key player in the disruption of endothelial homeostasis that promotes cell hypomethylation and modulate epigenetic mechanisms, by compromising DNA and histone methylation [30,31,32,33]. We demonstrated a specific mechanism by which SAH-induced endothelial hypomethylation up-regulates the expression of the adhesion molecules ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular adhesion molecule-1) [30]. During atherogenesis, the inflammatory activation of endothelial cells is characterized by the upregulation of adhesion molecules, which by recruiting circulating leukocytes favor their trans-endothelial migration and atherosclerosis progression. In a series of studies, we demonstrated that SAH- induced up-regulation of adhesion molecules promoted leukocyte adhesion [30]. Further, we documented the ability of excess SAH to activate endothelial prototypical pro-inflammatory signaling pathways and to up-regulate the expression of several cytokines including TNF α (tumor necrosis factor alpha) [31]. Moreover, our studies lead to the discovery that a specific methylation modification of histones is also decreased by endothelial hypomethylation contributing to endothelial dysfunction and activation. Interestingly, associations between altered methylation status of histone and changes in AQPs expression have been observed in different cell models [34,35].

In this study, we used human cultured endothelial cells to confirm the ability of excess SAH to trigger a pro-atherogenic phenotype and investigate the expression of several adhesion molecules at different cellular localizations, including at the cell surface. Subsequently, by performing a trans-endothelial cell migration assay, we attested the functional importance of SAH-mediated up-regulation of adhesion molecules. Moreover, we measured cell membrane water and glycerol permeability in the presence and absence of SAH-induced hypomethylation. In order to ascertain a contribution of altered AQP-expression in impaired cell permeation and endothelial

dysfunction, we evaluated the expression levels of several orthodox AQPs and aquaglyceroporins. Lastly, to gain insights into the potential molecular mechanisms that underlie the association between SAH-induced cell activation and altered AQPs expression, we investigated the role of exogenous TNF α on the expression of the most abundant endothelial AQPs.

Results

SAH-induced endothelial dysfunction

Excess SAH induces cell hypomethylation. In order to promote SAH intracellular accumulation, the activity of the enzyme responsible for its production, SAH hydrolase, was inhibited with the adenosine analog, ADA. A strong intracellular accumulation (by more than 6-fold) of SAH in HUVEC exposed to ADA associated with a cell activation phenotype has been previously observed by us [30,32]. Here, and accordingly, ICAM-1, VCAM-1, and E-selectin mRNA levels were increased in ADA-treated cells (Figure 2.1A). Additionally, an up-regulation of the same adhesion molecules at the cell surface was observed (2.1 ± 0.5 , 1.3 ± 0.5 , and 1.2 ± 0.1 -fold increase of ICAM-1, VCAM-1, and E-selectin, respectively ($P < 0.05$) (Figure 2.1B). Finally, to attest the functional importance of the SAH-mediated up-regulation of adhesion molecules, we performed a trans-endothelial cell migration assay. Our results revealed a 2.2 ± 1.3 -fold increase in the number of leukocytes that migrated through the endothelial cells when they were previously treated with ADA ($P < 0.05$ versus control; Figure 2.1C).

Effect of hypomethylation on water and glycerol permeability

A recent study showed that AQP1 is expressed in human atherosclerotic vascular lesions and its normal water channel permeability was considered important to promote cardiovascular protection by maintaining endothelial water homeostasis [15], but there are no reports regarding aquaglyceroporin and glycerol permeability. This prompted us to evaluate membrane permeability to water (P_f) and to glycerol (P_{gly}) in human endothelial cells, under normal conditions or ADA-induced hypomethylating environment. Permeability was assayed in individual adherent cells loaded with calcein under a fluorescence microscope as previously described [3] (see Methods).

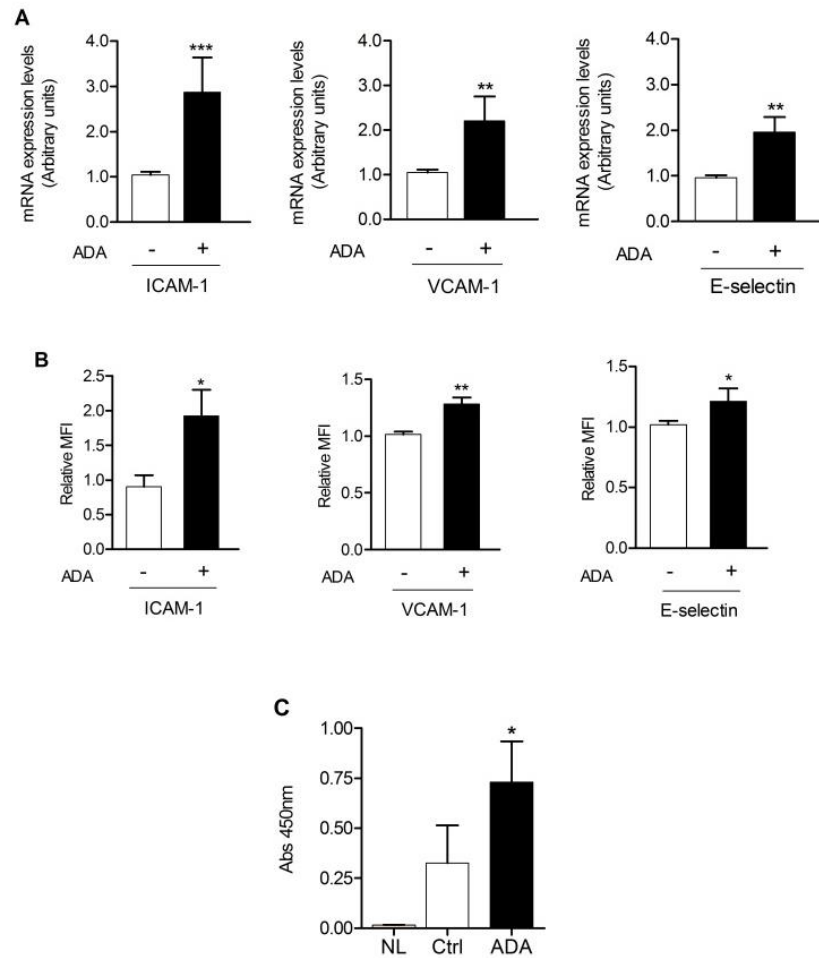


Figure 2.1. SAH accumulation and endothelial cell activation. (A) mRNA expression levels of ICAM1, VCAM1 and SELE were measured by quantitative RT-PCR to determine the effects of ADA compared to control (n=3-5). (B) Flow cytometry was used to detect the relative cell-surface expression of ICAM-1, VCAM-1, and E-selectin, in ADA-treated and non-treated HUVEC (n=3-6). MFI, mean fluorescence intensity. (C) The effect of ADA treatment on the number of leukocytes that migrate through the endothelial monolayer was analysed by a trans-endothelial migration colorimetric assay (n=3). NL, no leukocytes added. Absorbance was measured at 450 nm. Data represent mean $\pm$ SD from three independent experiments. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ADA versus non-treated cells.

When cells are exposed to a hyperosmotic mannitol buffer (impermeant solute), water outflow induces cell shrinkage (Figure 2.2A). Water permeability P_f is evaluated by monitoring the time course of fluorescence output that reflects the transient volume change (V_{rel}). As shown in Figure 2.2A, cells treated with ADA show slower rate of volume equilibration when compared with control cells. The calculated P_f values of cells in endothelial dysfunction condition ($(4.99 \pm 0.5) \times 10^{-3} \text{ cm s}^{-1}$) was significantly

lower than control non-treated ($(6.29 \pm 0.6) \times 10^{-3} \text{ cm s}^{-1}$) (Figure 2.2B), suggesting that water channel proteins are involved in disease.

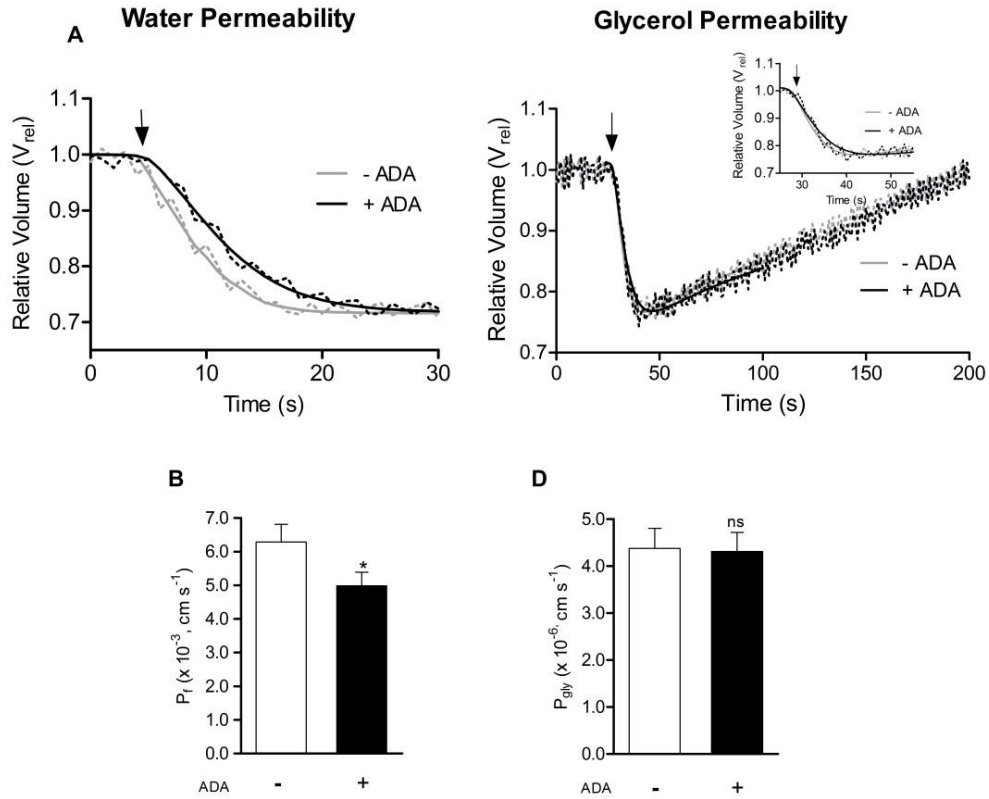


Figure 2.2. Water and glycerol transport in HUVEC under normal or hypomethylating environment. Water and glycerol permeability were assayed in adherent endothelial cells grown without (-) and with ADA (+). **(A)** Time course of cell volume change caused by an osmotic challenge with mannitol (arrow) ($n=10$). **(B)** Water permeability (P_f). **(C)** Time course of cell volume change caused by an osmotic challenge with glycerol (arrow) ($n=10$). Inset shows the first 50 s of cell volume change after glycerol addition. **(D)** Glycerol permeability (P_{gly}). Bars show mean $\pm$ SD from 10 cells analysed on 3 coverslips in 2 cell platings. ns, not significant, *, $P < 0.05$ ADA versus non-treated cells.

To assess glycerol permeability P_{gly} , cells were challenged with a glycerol hyperosmotic solution and the time course of cell volume equilibration (V_{rel}) was monitored. After the first fast cell shrinkage due to water outflow, glycerol influx is followed by water with consequent cell reswelling [3] (Figure 2.2C). ADA-treated and control cells showed similar rates of glycerol influx and no differences were detected. The calculated P_{gly} for cells in hypomethylation condition ($(4.3 \pm 0.5) \times 10^{-6} \text{ cm s}^{-1}$) was similar to that measured for control non-treated cells ($(4.4 \pm 0.5) \times 10^{-6} \text{ cm s}^{-1}$) (Figure 2.2D), indicating that SAH accumulation does not affect glycerol permeability. Though not affected by hypomethylation, these permeability values are within the

range of the P_{gly} measured in other mammalian cell lines with high endogenous expression of aquaglyceroporin ((2 to 5) $\times 10^{-6}$ cm s⁻¹), such as AQP7 and AQP3 in mouse adipocytes [36,37], rat pancreatic β -cells [38] and human epidermoid carcinoma cells [39] respectively, and contrasting with the low P_{gly} ((0 to 0.2) $\times 10^{-6}$ cm s⁻¹) found in cells with residual aquaglyceroporin level [39,40]. Thus, the P_{gly} values evaluated may reflect the contribution of aquaporins in HUVEC for glycerol transport.

AQP3 is the main endothelial aquaglyceroporin

To investigate if AQPs are involved in water and glycerol transport in human endothelial cells we first examined the gene expression levels of the aquaglyceroporins AQP3, 7, 9 and 10 in HUVEC (Figure 2.3A). Our results show that AQP3, AQP7 and AQP10 mRNAs, but not AQP9 mRNA, are present in HUVEC. Moreover, we found that AQP3 was the predominant aquaglyceroporin gene present in the human endothelium.

Under SAH accumulation, AQP3 mRNA expression was not affected (0.931 ± 0.1 -fold), while AQP7, the less expressed aquaglyceroporin, was upregulated (6 ± 1.2 -fold; $P < 0.001$) and AQP10 was downregulated (0.37 ± 0.02 -fold; $P < 0.001$). Thus, the overall expression of aquaglyceroporins seems not to be affected by ADA, since AQP3 gene expression does not change and although AQP7 is upregulated, the opposing AQP10 downregulation compensates the still very low AQP7 upregulated levels (Figure 2.3B). However, since we obtained P_{gly} values supporting aquaglyceroporin activity and AQP3 is the main aquaglyceroporin expressed in HUVEC, we subsequently evaluated AQP3 protein expression in control and ADA-treated cells (Figure 2.3C and 3D). The effect of ADA in AQP3 protein level (1.38 ± 0.6 -fold) is in line with the AQP3 mRNA expression and discards any effect of SAH accumulation. Thus, our data show that AQP3 is the main glycerol channel in endothelial cells responsible for endothelium glycerol permeation; however, despite its physiological role in glycerol permeation, AQP3 may not be involved in the development of a vascular pathology.

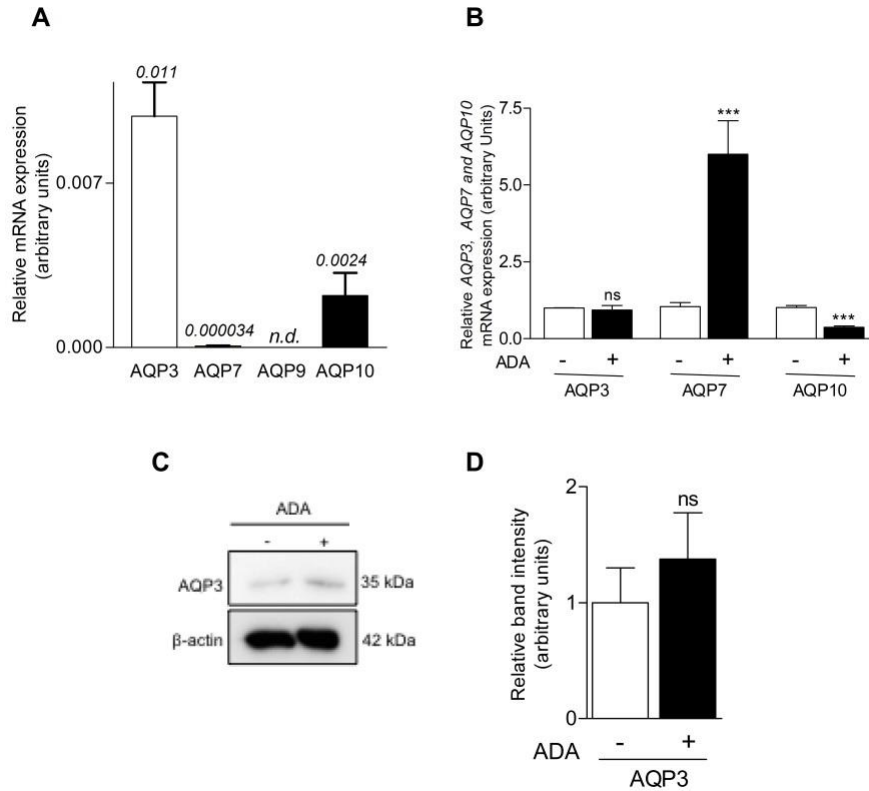


Figure 2.3. Effect of SAH accumulation on aquaglyceroporins expression in HUVEC. (A) AQP3, AQP7, AQP9 and AQP10 mRNA expression levels. **(B)** AQP3, AQP7 and AQP10 mRNA expression levels in endothelial cells grown without (-) or with ADA (+). Aquaporin mRNA levels are relative to β -actin mRNA levels and normalized to untreated expression for each AQP. **(C)** Representative blots of AQP3 protein in endothelial cells obtained from HUVEC cultures non-treated (-) and treated (+) with ADA. **(D)** Relative AQP3 protein expression. Data represent mean $\pm$ SD from three independent experiments. *n.d.*, not detected; ns, not significant, ***, $P < 0.001$ ADA versus non-treated cells.

AQP1 is implicated in endothelial dysfunction

AQP1 was reported the most expressed aquaporin in human endothelial cells [13]. In view of the decreased P_f obtained when HUVEC cells were treated with ADA, we evaluated AQP1 mRNA and protein levels in ADA treated and control cells. In addition, we also investigated AQP5 mRNA expression in HUVEC since AQP5 is widely distributed in human tissues and its abnormal expression and function were correlated with several disease conditions [41]. Semi-quantitative gene expression of these AQPs (Figure 2.4A) confirmed AQP1 as the most expressed aquaporin in endothelium and also detected AQP5 mRNA expression although in a much lower amount.

The pro-inflammatory mediator TNF α is implicated in the regulation of the expression levels of the AQP1 and AQP5 [42,43]. Interestingly, we recently reported a link between ADA-induced hypomethylation and the increase of TNF α levels in cultured human endothelial cells [31]. This led us to investigate the role of TNF α in the ADA-induced downregulation of the AQP1 and AQP5 expression. The results, depicted in Figure 2.1.4B, show that TNF α treatment downregulates the expression of both AQP1 and AQP5 (0.183 ± 0.01 and 0.083 ± 0.01 -fold $P<0.001$, respectively), as previously reported [42].

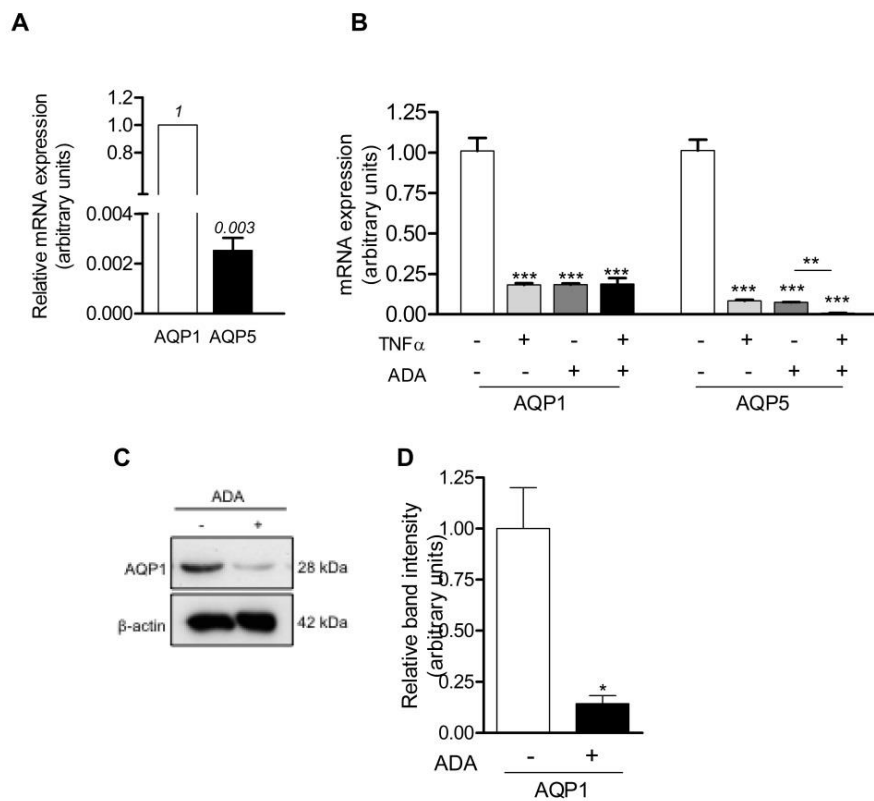


Figure 2.4. Effect of SAH and TNF α in AQP1 and AQP5 expression. (A) AQP1 and AQP5 mRNA expression levels in HUVEC. Aquaporin mRNA levels are relative to the mean of β -actin. (B) AQP1 and AQP5 mRNA expression levels in endothelial cells grown without (-) or with ADA (+) and without (-), or with TNF α (+). The effect of TNF α was achieved by culturing the cells in the presence of 10 ng/mL TNF α for 24h. (C) Representative blots showing AQP1 protein in endothelial cells obtained from HUVEC cultures non-treated (-) and treated (+) with ADA. (D) Relative AQP1 protein expression. Data represent mean $\pm$ SD from three independent experiments. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ versus non-treated cells.

In addition, the expression levels of AQP1 and AQP5 were also drastically decreased under SAH accumulation conditions (0.184 ± 0.02 and 0.074 ± 0.01 -fold;

$P < 0.001$, respectively). However, co-incubation with $\text{TNF}\alpha$ and ADA did not affect the already decreased AQP1 expression (0.187 ± 0.05 -fold; $P < 0.001$), whereas AQP5 was further downregulated (0.0062 ± 0.002 -fold; $P < 0.001$).

The role of AQP1 as main endothelial water channel was confirmed by evaluating AQP1 protein expression in HUVEC cells. As shown in Figure 2.4C and 4D, AQP1 protein expressed in control cells was dramatically reduced when cells were treated with ADA, in agreement with the obtained mRNA expression levels and with the measured water permeability in control and hypomethylation environment. These results strongly suggest that besides being the main water channel, AQP1 is involved in endothelial dysfunction.

Discussion

Aquaporins are crucial for the maintenance of tissue homeostasis. Previous studies have shown the involvement of AQPs in vascular pathophysiology. Recently, based on immunolabel-based detection approaches, a few aquaporin isoforms were found in mammalian vascular tissues [12,21,22,23,24] but their exact location in endothelial cells has not been ascertained. Here, by analyzing AQPs gene expression, we report for the first time that AQP3 is the most expressed aquaglyceroporin in primary human endothelial cells followed AQP10 and AQP7 in much lesser amounts. Accordingly, the permeability values for water and glycerol transport are in the range of those reported for other mammalian cells with high endogenous aquaglyceroporin expression [36,39].

This study was designed to investigate the potential involvement of aquaglyceroporins in endothelial dysfunction as a critical step in the development of vascular pathologies such as atherosclerosis. Impaired methylation has been associated with several diseases, including CVD [29,44,45,46,47]. Our previous studies using the same cell model have shown that an altered methylation status, due to excess SAH, promotes endothelial dysfunction by impairing nitric oxide (NO) bioavailability and by altering the cellular redox status [30,33,45]. Additionally, we found that excess SAH can lead to endothelial inflammation, inducing the expression of adhesion molecules and inflammatory cytokines [31]. Here, we examined the endothelial expression of key cell-surface proteins involved in the leukocyte adhesion and transmigration processes (Figure 2.1). ICAM-1, VCAM-1 and E-selectin contribute to the early stages of leukocyte adhesion [48]. We found that excess SAH increases the cell surface

expression of these adhesion molecules, confirming our previous findings [30,31], that ICAM-1, VCAM-1 and E-selectin mRNA levels are increased in endothelial cells following ADA treatment. Moreover, we attested the functional importance of ADA-induced up-regulation of adhesion molecules by performing a trans-endothelial migration assay that revealed a two-fold increase in the number of leucocytes that migrated through ADA treated cells, in comparison to non-treated cells. This observation discloses a new association between endothelial hypomethylation and augmented endothelial transmigration, a key feature of atherosclerosis development.

Water and glycerol permeability in ADA-treated and non-treated cells (Figure 2.2) was then studied. Our results show that endothelial hypomethylation is associated with altered water transport (20% decrease of P_f), while glycerol permeability remains unaltered. This observation suggests that orthodox AQPs function and/or expression is impaired under endothelial hypomethylation, with potential implications for the establishment of a pro-atherogenic endothelial phenotype. As we found AQP1 and AQP3 to be the most predominant in HUVEC (Figure 2.4A and 3A, respectively), we further assessed their expression under a hypomethylating environment. We observed a decreased expression of AQP1 but not of AQP3 in ADA-treated cells versus control (Figure 2.4B and 3B, respectively). Importantly, there is a correlation between mRNA and protein levels for both AQPs (Figure 2.3 and 4). In fact, AQP1 was previously reported to play a relevant role in the control of water permeability in endothelial cells [49]. Moreover, AQP3, although being a functional aquaglyceroporin that contributes to endothelium glycerol permeation, seems not to contribute to the development of ADA-induced endothelial dysfunction.

The observed changes in AQP1 expression in response to increased SAH levels may rely on altered transcription rates or mRNA stability. A recent study reported that AQP1 is intimately associated with non-activated, atheroprotected, endothelium whereas expression is absent in activated, ICAM-1 positive endothelium [14]. AQP1 expression is highly regulated and its promoter includes different regulatory elements, such as the atheroprotective transcription factor KLF2 [14], Sp1, AP1, AP2, and E-box elements [50]. It was reported that AQP1 expression can be reduced in the presence of TNF α or IL-1 β cytokines in rat endothelial cells [43], and that TNF α might regulate AQP1 and AQP5 expression in a NF- κ B dependent manner in human epithelial cells [42]. Interestingly, a previous study focused on AQP5 expression reported the first link between aquaporin expression and NF- κ B activation [51]. The authors reported that

TNF α effect in AQP5 expression requires the nuclear translocation of NF- κ B. The mechanism by which NF- κ B activation downregulates AQP5 could not be clarified, however it was suggested that (i) a direct interaction with the AQP promoter might occur, where NF- κ B may function as a transcriptional repressor. Alternatively, (ii) p50 and p65 NF- κ B subunits can repress transcription by direct interaction with other transcription factors, such as SP1 [51].

Our group has previously reported that SAH-induced hypomethylation leads to NF- κ B activation, which mediates the increased expression of adhesion molecules and inflammatory cytokines, as TNF α and IL-1 β [52]. In order to further dissect the mechanism behind SAH intracellular accumulation and AQP1 downregulation, we assessed the AQP1 and AQP5 endothelial expression in the presence of ADA, TNF α , or both (Figure 2.4B). Independent treatments with ADA or TNF α resulted in a similar effect, down-regulation of both AQP1 and AQP5. However, co-incubation only showed a cumulative effect with AQP5, suggesting that ADA-mediated regulation of AQP1 may occur exclusively through TNF α . Accordingly with our previous results, increased TNF α in cells with increased SAH levels is dependent on NF- κ B activation, but whether NF- κ B mediates AQP1 transcriptional repression remains to be investigated.

Osmotic stress is one of the most challenging trials experienced by living cells and in order to maintain homeostasis, cells have to adapt fast when confronted with environmental perturbations. Aquaporins are among the mechanisms developed by living organisms to overcome these stresses. Since aquaporins are facilitators of membrane water permeation along with the much slower passive diffusion, and in endothelial dysfunction AQP1 is downregulated as a consequence of SAH accumulation and hypomethylation environment, we can infer that this disease condition renders cells more sensitive to osmotic stress. Notably, AQP1 was also reported to transport NO, the main anti-atherogenic molecule that plays a key role in the protection against the onset and progression of cardiovascular disease [53]. Therefore, AQP1 downregulation impact on endothelial function may go beyond osmotic transport impairment and further studies are required to better clarify its role in vascular disease.

Conclusions

Reduced AQP1 levels were previously associated with different CVD features, as ischemia, hypoxia, and cardioplegia [52]. Our results support the involvement of impaired AQP1-mediated water transport in endothelial dysfunction and activation.

The homocysteine precursor, SAH, is once more shown as an important player in endothelial homeostasis regulation. Our results suggest an additional mechanism through which SAH-induced hypomethylation environment may contribute to an endothelial pro-atherogenic phenotype, further supporting its causal role in vascular pathology.

We have found that increased SAH in human endothelial cells leads to decreased AQP1 levels and altered water permeability, with no effects on AQP3 levels and glycerol permeability. Furthermore, our results show that TNF α treatment downregulates AQP1 expression in endothelial cells, suggesting that SAH-induced inflammatory activation might be responsible for the observed SAH-mediated decrease of AQP1. Although further investigation is needed to validate this hypothesis, our results suggest that AQP1 is a candidate player in the setting of endothelial dysfunction.

Materials and Methods

Cell Culture and Treatments

Human umbilical vein endothelial cells (HUVECs) obtained from Lonza (Walkersville, MD, USA) were cultured at 37 °C in 5% CO₂. Cells were grown in EGM-2 supplemented medium (Lonza). Experiments were performed between passages two and five and with cells 70 to 80% confluent. Treatments with adenosine-2',3'-dialdehyde (ADA; Sigma) were performed for 48 h at 20 μ M as previously optimized [54]. Tumor Necrosis Factor- α (TNF α) treatments (10 ng/mL; Sigma) were performed for 24 h.

RNA extraction and quantitative PCR

Total RNA was isolated using a RNeasy mini kit (Qiagen) and treated with DNase I (Invitrogen). cDNA was obtained from 500 ng of total RNA and the retrotranscription reaction was carried out with SuperScript III kit (Invitrogen). Real-time PCR reactions were carried out using a CFX96 Real-Time System C1000 (BioRad), the TaqMan Universal PCR Master Mix (Applied Biosystems) and the following specific TaqMan pre-designed gene expression primers: AQP1 (Hs01028916_m1), AQP3 (Hs01105469_g1), AQP5 (Hs00387048_m1), AQP7

(Hs00357359_m1), AQP9 (Hs00175573_m1), ACTB (Hs99999903_m1), ICAM-1 (Hs00164932_m1), VCAM-1 (Hs00365485_m1) and SELE (Hs00950401_m1) (Applied Biosystems). The delta-delta-Ct method was used for relative quantification of gene expression, using β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous controls.

Flow cytometry

Flow cytometry was used to assess the expression of adhesion molecules in the cell surface. Adhesion molecules were stained independently with specific fluorescent-conjugated antibodies for ICAM-1 (SantaCruz Biotechnology), VCAM-1 (R&D Systems) and E-selectin (BioLegend). Briefly, cells were trypsinized, washed with PBS, incubated for 30 min with specific antibodies, washed with PBS containing 0.1% NaN₃, and analyzed using GUAVA EasyCyte 5HT FACS and InCyte software (Millipore).

Transmigration assay

Transendothelial migration of leukocytes was assessed using the QCM™ leukocyte transendothelial migration colorimetric assay (Millipore). Briefly, cells (80-90% confluent) were cultured in the presence or absence of ADA, for 24 hours, and then replaced onto fibronectin-coated cell culture inserts (3 μ m pore size) maintaining culture conditions. After 24h, fresh leukocytes isolated from healthy individuals, that were processed with an RBC lysis solution (Citogene) and re-suspended in DMEM media (Life Technologies) containing 0.5% BSA (Sigma), were added to the insert, on the top of the confluent HUVEC. After 8 hours of incubation, the leukocytes in the lower chamber, which migrated across the endothelial cell monolayer, were stained accordingly to assay instructions and measured at 450nm using an Asys Expert Plus plate reader (Biochrom).

Western Blotting

Proteins were separated by SDS-PAGE before semi-dry transfer. After blocking, the following specific primary antibodies were used: anti-AQP1 and AQP3 antibody (1:100 and 1:200, respectively; Santa Cruz Biotechnology), and anti- β -actin (1:1000; Sigma–Aldrich). Secondary anti-rabbit and anti-mouse (1:5000; Jackson Immuno Research) and anti-goat (1:7500; Jackson Immuno Research) HRP-linked antibodies were used for ECL-mediated (Amersham Biosciences) detection. Band intensity was measured using the ImageJ software (<https://imagej.nih.gov>).

Permeability assays

Water (P_f) and glycerol (P_{gly}) permeability were measured in individual adherent cells on a coverslip, as previously described [37,55]. Briefly, cells were loaded with 5 mM calcein acetoxymethyl ester (calcein-AM; Sigma-Aldrich) for 30 min at 37°C in 5% CO₂. The coverslips with the adhered cells were mounted in a closed perfusion chamber (Warner Instruments, Hamden, USA) on the stage of a Zeiss Axiovert 200 inverted microscope. Fluorescence was excited at wavelength 495/10nm and the emission fluorescence collected with a 535/25 nm bandpass filter coupled with a 515nm dichroic beam splitter. Images were captured using a ×40 epifluorescence oil immersion objective and a digital camera (CoolSNAP EZ, Photometrics, USA) and recorded by the Metafluor Software (Molecular Devices, USA).

Cells were perfused with 300 mM HEPES buffer (135 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D-Glucose, 5 mM HEPES, pH 7.4, 300 mOsM) for 60 s, after which 300 mM mannitol (for water permeability) or 300 mM glycerol (for glycerol permeability) was added to the buffer to achieve an external osmolarity of 600 mOsM. Cell volume (V) was measured at selected time points from 2D images obtained during the permeability assays to evaluate the initial volume (prior to the osmotic challenge V_o) and the final equilibrium volume [37]. P_f and P_{gly} coefficients were evaluated from the measured time dependent volume changes, $V_{rel} = V/V_o$, obtained by adding mannitol (P_f) or glycerol (P_{gly}) to the external media, using the model equations described by Madeira et al. [36] and the Berkeley Madonna software (<http://www.berkeleymadonna.com>).

Statistical analysis

Results were expressed as mean ± SD of n individual experiments. Statistical analysis between groups was performed by Student's t -test. P values <0.05 were considered statistically significant. Statistical analyses were performed using the Graph Prism software (GraphPad Software).

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2.2. Involvement of aquaporins in obesity-induced inflammation in endocrine pancreas

This subchapter is adapted from the following manuscripts:

Inês Vieira da Silva, Leire Mendez-Gimenez, Carlos Cardoso, Sergio Póvoa Camoes, Gema Fruhbeck, Amaia Rodriguez, Joana Paiva Miranda and Graça Soveral. Involvement of pancreatic aquaporin-7 and -12 in obesity-induced inflammation (*in preparation*)

Leire Mendez-Gimenez, Sara Becerril, Sergio Póvoa Camoes, **Inês Vieira da Silva**, Cláudia Rodrigues, Rafael Moncada, Víctor Valentí, Vitoria Catalan, Javier Gomez-Ambrosi, Joana Paiva Miranda, Graça Soveral, Gema Fruhbeck and Amaia Rodriguez. Role of aquaporin-7 in ghrelin- and GLP-1-induced improvement of pancreatic beta-cell function after sleeve gastrectomy in obese rats. *International Journal of Obesity* (Lond) (2017) 41: 1394-1402. DOI: 10.1038/ijo.2017.135

Abstract

Aquaporins (AQPs) are membrane channels that facilitate water and glycerol movement across cell membranes. Recently, the water and glycerol channel AQP7, the most representative aquaporin in pancreatic β -cells, was suggested to play a role in insulin exocytosis, thus contributing to energy homeostasis. AQP12, whose selectivity and function are still unclear, was previously shown to be upregulated in pancreatitis. However, few is known about the implication of these two isoforms in endocrine pancreas-associated pathologies, such as Type-2 Diabetes (T2D) and insulin resistance (IR). Here, we investigated the contribution of AQP7 and AQP12 to obesity-induced inflammation in endocrine pancreas using a loss-of-function/gain-of-function strategy. For that, we assessed AQP7 and AQP12 expression in healthy and in pro-inflammatory phenotypes. The cell model was validated by evaluating AQP7 and AQP12 mRNA and protein expression as well as their activity as water and glycerol channels. Their implication in inflammation was evaluated by assessing gene expression levels as well as markers of inflammation in a $\text{TNF}\alpha$ - or LPS-induced pro-inflammatory phenotype. Our data confirms AQP7 as a functional water and glycerol channel in pancreatic β -cells with implications in several physiological processes, such as by increasing cell proliferation, migration and adhesion. As expected by its intracellular localization, AQP12 function was not detectable in the plasma membrane. Contrary to $\text{TNF}\alpha$, LPS induces AQP7 upregulation. Both increase and decrease in AQP7 expression seems to have a negative effect in insulin secretion in $\text{TNF}\alpha$ induced inflammation. On the other hand, both $\text{TNF}\alpha$ and LPS upregulate AQP12. $\text{TNF}\alpha$ -induced inflammation impairs AQP7 expression and drastically reduces insulin secretion. Importantly, cells overexpressing AQP12 are more resistant to $\text{TNF}\alpha$ - or LPS- induced inflammation, revealing lower levels of proinflammatory markers. Altogether, these data clearly document AQP7 involvement in insulin secretion and AQP12 implication in inflammation, being their expression levels fundamental for pancreatic β -cells physiological function.

Keywords: Aquaporins, Aquaglyceroporins, Glycerol, Pancreatic β -cells, Inflammation, Obesity, Type-2 Diabetes

Introduction

Aquaporins (AQPs) are channel-forming integral membrane proteins involved in the transport of water and small molecules across plasma membrane driven by osmotic or solute gradients [1,2]. These channels are widely distributed among all kinds of organisms and play a variety of important physiological roles [3]. The 13 isoforms detected in humans (AQP0-12) are divided into three subgroups based on their structural and functional properties: orthodox aquaporins (AQP0, 1, 2, 4, 5, 6 and 8) which are selective water channels, aquaglyceroporins (AQP3, 7, 9 and 10) which also transport other small uncharged solutes (such as glycerol and urea) and S-aquaporins (AQP11 and 12) whose selectivity is still controversial [4,5].

Adipose tissue produces adipocytokines, which flow through the bloodstream, reaching peripheral tissues where they exert specific effects on metabolism. Adipose tissue of obese and type 2 diabetic individuals is infiltrated by resident macrophages and is in a state of chronic inflammation [6,7]. The adipocytes and infiltrated macrophages secrete proinflammatory cytokines (TNF α , IL-6, IL-1 β) that promote insulin resistance [8]. Several studies have demonstrated that cytokines also increase adipose lipolysis affecting the stability of lipid droplets in adipocytes by influencing the proteins that stabilize the lipid droplets [9,10]. TNF α decreases perilipin expression, presumably enhancing the ability of lipases to access triglyceride within the lipid droplets, increasing lipolysis [11]. In addition to proinflammatory cytokines, excess lipolysis drives high amounts of saturated fatty acids to ectopic lipid accumulation further contributing to pancreatic β -cell dysfunction and leading to impairment in insulin signaling. Most recently, Toll-like receptors (TLR), whose stimulation typically leads to activation of proinflammatory processes in response to microbial pathogens, were shown to constitute a molecular link between elevated circulating saturated FFA and underlying inflammation in insulin-sensitive tissues [12]. In fact, saturated long chain FFA alone are capable of activating TLR2 and TLR4 [13].

Aquaglyceroporins are emerging as key players in body energy homeostasis with potential implications in obesity and related metabolic complications, such as T2D and IR [14,15,16]. In circumstances of negative energy balance, glycerol produced by TAG lipolysis is released by the adipocyte via AQP7 into the bloodstream, flowing to peripheral tissues to be used as energy substrates [17]. Circulating glycerol constitutes an important energy substrate during fasting with the liver being responsible for about

70–90% of whole-body glycerol metabolism [18]. AQP9 constitutes the main route for hepatocyte glycerol uptake [19,20]. The close coordination between adipose and hepatic aquaglyceroporins is required for the control of whole-body glucose homeostasis as well as lipid accumulation [14,21,22].

AQP7 is also the most representative glycerol channel expressed in pancreatic β -cells, endocrine cells responsible for insulin production, and thus, regulating the blood glucose uptake [23,24,25]. Glycerol uptake is followed by a fast β -cell reswelling, volume-regulated anion channels activation and insulin release [23]. Glycerol kinase phosphorylates glycerol to glycerol-3-phosphate, which is used as a substrate in the glycerol-3-phosphate shuttle, the process that reduces equivalents into mitochondria to be used in oxidative phosphorylation and ATP production [25,26,27]. The increase in the cytosolic ATP:ADP ratio triggers the closure of ATP-sensitive K^+ channels, cell membrane depolarization and opening of voltage-sensitive Ca^{2+} channels, ultimately leading to the exocytosis of insulin-containing granules and insulin secretion [23,28,29]. Interestingly, AQP7-deficient mice develop adult-onset obesity, insulin resistance and hyperinsulinemia [30]. In this regard, AQP7-knockout mice show increased β -cell glycerol content and glycerol kinase activity, which results in higher basal and glucose-induced insulin secretion as well as reduced β -cell mass indicating a more efficient insulin biosynthesis and secretion [25,30].

On the other hand, AQP12 is expressed in pancreatic acinar cells and localizes on the membrane of intracellular organelles [31,32]. The intracellular localization of this S-aquaporin in pancreatic acinar cells suggests a potential role in the maturation and exocytosis of zymogen granule [31]. Data based on AQP12-null mice showed increased susceptibility to acute pancreatitis, probably due to the defect in the secretion of zymogens [32]. However, its potential expression and function in β -cells remains unknown.

The impact of obesity-induced inflammation in pancreatic aquaporins has not been completely clarified yet. In this study, we used rat pancreatic β -cells (RIN-m5F cell line) to confirm the ability of exogenous $TNF\alpha$ to trigger a pro-inflammatory phenotype, using a loss-of-function/gain-of-function strategy. First, we verified the presence of AQP7 and AQP12 at transcript and protein levels and measured cell membrane water and glycerol permeability to attest aquaporin function in this cell line. Subsequently, we generated cell lines overexpressing and silenced for AQP7 and AQP12 in order to investigate the role of AQP7 and AQP12 in the development of a

pro-inflammatory state. To evaluate the importance of AQP7 and AQP12 in physiological processes, we performed proliferation, migration and adhesion assays. Furthermore, we aimed at unraveling the implication of AQP7 and AQP12 in inflammation in endocrine pancreas, since their expression is altered in this condition.

Results

RIN-m5F cell line naturally expresses AQP7 and AQP12

Pancreatic aquaglyceroporin transcript levels were previously evaluated in rat tissues where AQP7 was identified as the most abundantly expressed aquaglyceroporin, while AQP9 was only detectable in few samples [33]. In the same tissues, AQP12 expression was also detected at both transcript and protein levels. In RIN-m5F cell line, we detected AQP7 in higher amounts than AQP12 at both transcript (AQP7, $(4.90 \pm 1.29) \times 10^{-8}$; AQP12, $(5.12 \pm 1.08) \times 10^{-10}$) and protein levels (AQP7, 1.25 ± 0.08 ; AQP12, 0.35 ± 0.01) (Figure 2.5A, B and C). AQP7 facilitates glycerol permeation in β -cells with implications in insulin release [25], thus, we confirmed the water and glycerol permeability of the RIN-m5F β -cell line, based on its high insulin secretion rate [34]. The time course of the relative cell volume change (V_{rel}) of RIN-m5F β -cells exposed to an osmotic shock with mannitol, inducing water outflow and cell shrinkage, is shown in Figure 2.5D. The osmotic water permeability coefficient (P_f) for this cell line was $(0.94 \pm 0.10) \times 10^{-3} \text{ cm s}^{-1}$ (Figure 2.5E). Glycerol permeability was also evaluated by following cell volume changes of cells subjected to an osmotic shock with glycerol (Figure 2.5D). After shrinkage, due to water outflow, RIN-m5F cells reswell due to the AQP-facilitated glycerol influx, with a P_{gly} of $(1.07 \pm 0.19) \times 10^{-6} \text{ cm s}^{-1}$ (Figure 2.5E). These permeability values are within the range those measured in other aquaporin-expressing cell lines, such as mice differentiated 3T3-L1 adipocytes (with endogenous AQP7 expression) [35] and human endothelial cells (with endogenous AQP3 expression) [36], and reflect the contribution of aquaporins for water and glycerol transport of RIN-m5F β -cells.

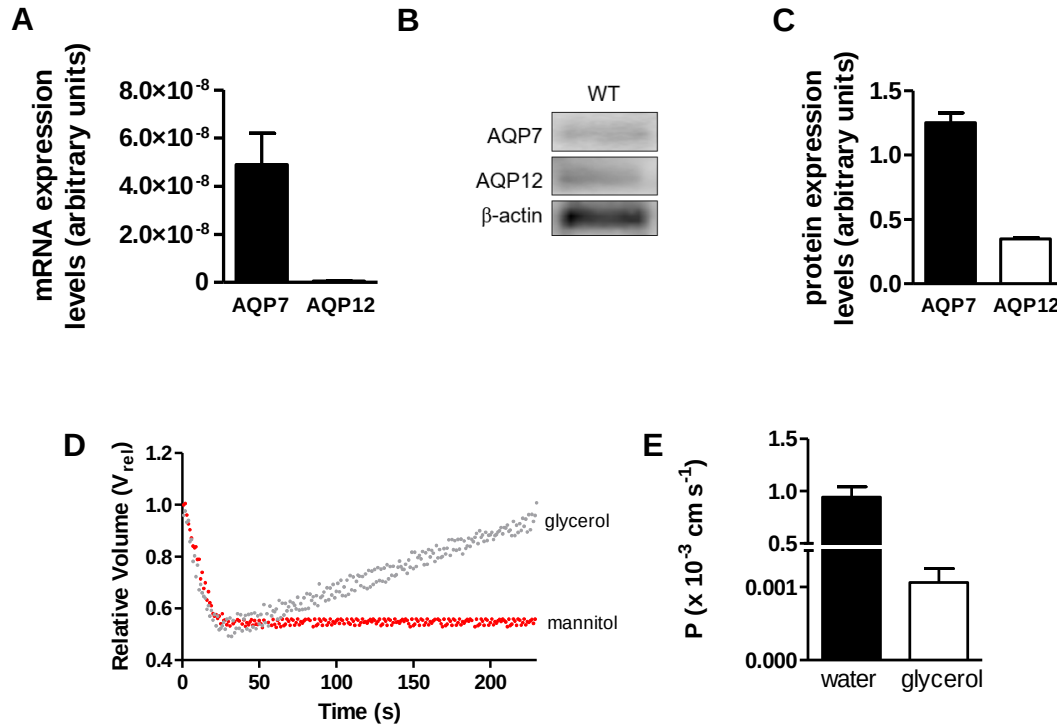


Figure 2.5. RIN-m5F cell line naturally expresses AQP7 and AQP12. mRNA (**A**) and protein (**B** and **C**) expression levels of AQP7 and AQP12 in RIN-m5F cell line. Values normalized to 18S for mRNA and to β -actin for protein relative expression. (**D**) Time course of cell volume changes caused by osmotic and solute challenges with mannitol (red dots) and glycerol (grey dots) in RIN-m5F cell line; (**E**) Water (P_f ; black bar) and glycerol permeabilities (P_{gly} ; white bar)

TNF α and LPS induce inflammation in pancreatic β -cell

In order to understand the involvement of pancreatic AQP7 and AQP12 in pancreatic β -cell function and impact in obesity induced inflammation, we evaluated the mRNA expression levels of AQP7 and AQP12 after treating cells with TNF α , one of the several proinflammatory cytokines released by adipose tissue of obese subjects, or with LPS, another ligand that can bind TLR4 similarly to FFA [8,13,37]. As illustrated in Figure 2.6A, AQP7 is downregulated by TNF α ($(1.00 \pm 0.16)^{-7}$) but upregulated by LPS ($(3.31 \pm 0.76) \times 10^{-9}$) comparing to non-treated cells ($(4.90 \pm 1.29) \times 10^{-8}$). On the other hand, AQP12 is upregulated by both TNF α ($(5.10 \pm 1.11) \times 10^{-9}$) and LPS ($(1.19 \pm 0.35) \times 10^{-8}$) comparing to control cells ($(5.12 \pm 1.08) \times 10^{-10}$). Transcript levels of proinflammatory cytokines, such as IL-6 (TNF α , $(2.18 \pm 0.53) \times 10^{-6}$; and LPS, $(5.81 \pm 1.10) \times 10^{-6}$), IL-1 β (TNF α , $(1.88 \pm 0.038) \times 10^{-7}$; and LPS, $(4.07 \pm 0.98) \times 10^{-8}$) and TNF α (TNF α , $(4.61 \pm 0.22) \times 10^{-5}$; and LPS, $2.14 \pm 0.20 \times 10^{-5}$), were upregulated in cells

treated with TNF α or LPS (Figure 2.6A). However, when evaluating the amount of protein release, IL-6 protein release increased (TNF α , 2.67 ± 0.07 pg mL $^{-1}$; and LPS, 2.37 ± 0.07 pg mL $^{-1}$; vs 2.00 ± 0.11 pg mL $^{-1}$ in non-treated cells). No variation in IL-1 β and TNF α release was observed with TNF α treatment, while IL-1 β was impaired (TNF α , 1.60 ± 0.20 pg mL $^{-1}$; and LPS, 0.82 ± 0.17 pg mL $^{-1}$; vs 1.60 ± 0.20 pg mL $^{-1}$ in non-treated cells) and TNF α release increased in LPS treatment (TNF α , 0.51 ± 0.01 pg mL $^{-1}$; and LPS, 1.12 ± 0.31 pg mL $^{-1}$; vs 0.51 ± 0.01 pg mL $^{-1}$ in non-treated cells) (Figure 2.6B). TNF α and LPS treatments were also correlated with the decreased release of anti-inflammatory cytokines. IL-4 (TNF α , 2.77 ± 0.15 pg mL $^{-1}$; LPS, 2.99 ± 0.01 pg mL $^{-1}$; vs 3.77 ± 0.27 pg mL $^{-1}$ in non-treated cells) was impaired in both treatments, while TGF β (TNF α , 157.54 ± 3.75 pg mL $^{-1}$; LPS, 184.25 ± 4.61 pg mL $^{-1}$; vs 191.54 ± 5.12 pg mL $^{-1}$ in non-treated cells) was impaired in TNF α -treated cells and IL-10 (TNF α , 1.2 ± 0.01 pg mL $^{-1}$; LPS, 0.762 ± 0.01 pg mL $^{-1}$; vs 1.2 ± 0.01 pg mL $^{-1}$ in non-treated cells) in LPS-treated cells (Figure 2.6B).

In addition, an impaired insulin release (TNF α , 18.44 ± 0.37 mU L $^{-1}$; LPS, 3.03 ± 0.36 mU L $^{-1}$ vs 122.94 ± 8.71 mU L $^{-1}$ in non-treated cells) was detected in both treatments and increased amounts of C-peptide (TNF α , 90.99 ± 1.51 pmol L $^{-1}$; LPS, 35.25 ± 0.79 pmol L $^{-1}$ vs 33.26 ± 1.48 pmol L $^{-1}$ in non-treated cells) were detected in the supernatants of TNF α -treated but not in LPS-treated cells (Figure 2.6B). Therefore, TNF α and LPS treatments seem to be good tools for further experiments concerning the effects of obesity-induced inflammation in pancreas and the role of aquaporins in this process.

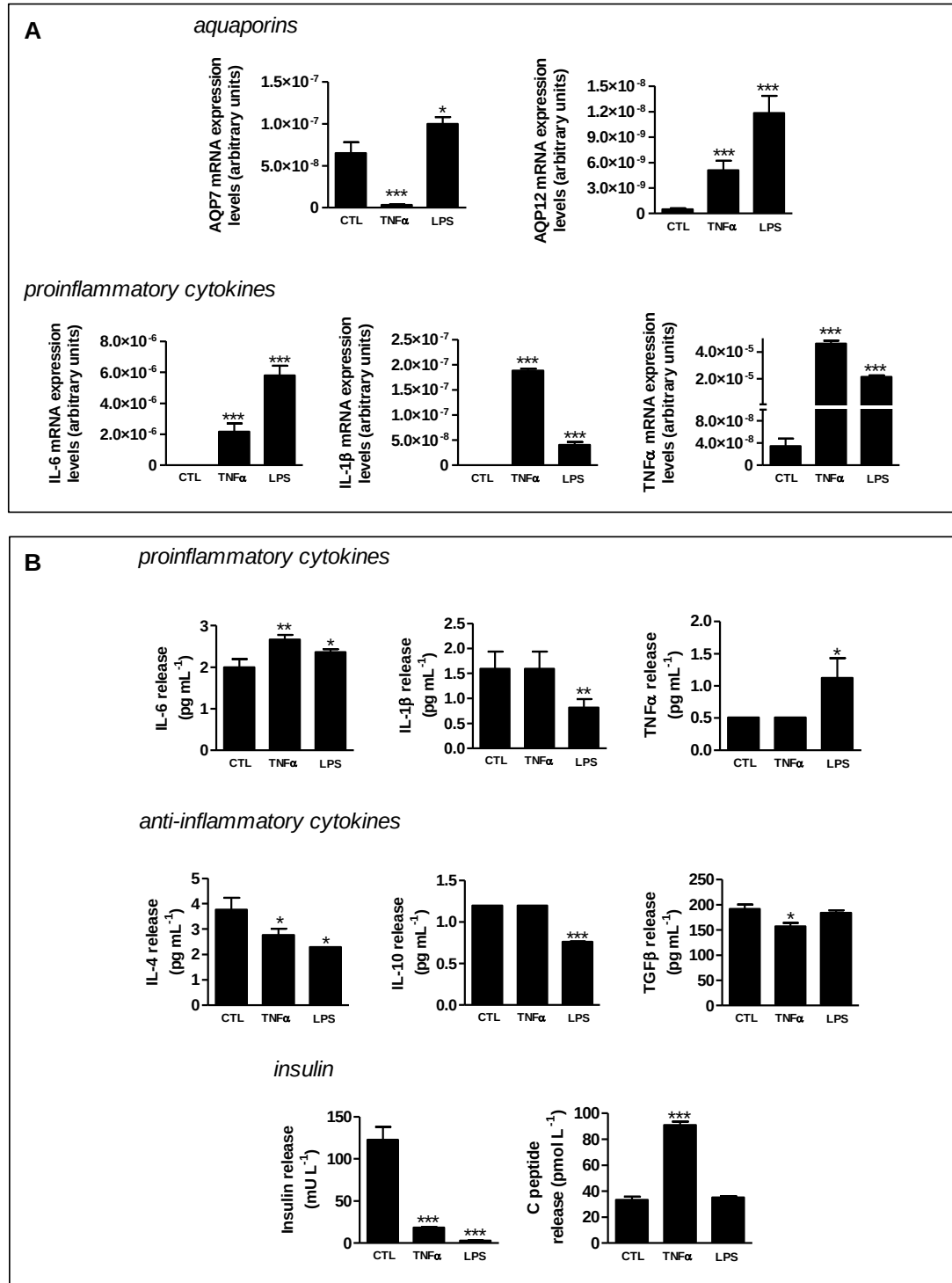


Figure 2.6. TNFα and LPS-derived inflammation affects AQP7 and AQP12 genes expression in RIN-m5F. **(A)** mRNA expression levels of AQP7 and AQP12 and proinflammatory cytokines IL-6, IL-1β and TNFα normalized to 18S in cells obtained from RIN-m5F cultures non-treated and treated with TNFα or LPS. **(B)** Protein release levels of proinflammatory cytokines (IL-6, IL-1β and TNFα), anti-inflammatory cytokines (IL-4, IL-10 and TGFβ), Insulin and peptide-C measured in supernatants from cells obtained from RIN-m5F cultures non-treated and treated TNFα or LPS to attest an inflammatory state. Data represent a mean of three independent experiments ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001; *, TNFα or LPS vs non-treated cells.

Validation of the experimental model gain of function/loss of function

To gain insights on the involvement of pancreatic AQP7 and AQP12 in the impairment of pancreatic function due to obesity, RIN-m5F cell lines overexpressing or silenced for each isoform were developed. Transfection controls for overexpression and silencing were produced in parallel. As shown in Figure 2.7A, AQP7 transfected clones showed significant increase in AQP7 expression ($(19.36 \pm 1.53) \times 10^4$ -fold, Figure 2.7A) and protein levels (1.63 ± 0.04 -fold, Figure 2.7B and C), when compared to wild type cells. On the other hand, shRNA transfected clones showed significant reduction of AQP7 expression levels (0.5 ± 0.07 -fold in mRNA; and, 0.31 ± 0.03 -fold in protein). Generated clones were also validated for their function as water and/or glycerol channels (Figure 2.7D and E). Our results showed that overexpressing AQP7 increases 1.35 ± 0.10 -fold the osmotic water permeability (P_f) and 1.60 ± 0.12 -fold the glycerol permeability. Instead, silencing AQP7 decrease P_f (0.55 ± 0.13 -fold) and P_{gly} (0.72 ± 0.03 -fold).

In addition, AQP12-overexpressing cells showed an increase of AQP12 mRNA and protein expression ($(14.07 \pm 9.53) \times 10^5$ -fold for mRNA; and 2.00 ± 0.02 -fold for protein), while cells silenced for AQP12 showed decreased levels of mRNA (0.26 ± 0.12 -fold) and protein (0.81 ± 0.06 -fold) (Figure 2.7F, G and H). As previously exposed, AQP12 is localized in intracellular membranes. Challenging whole cells with osmotic solutions of mannitol and glycerol, did not revealed AQP12 activity. Figure 2.7I and J, show the time course of cell volume change after the osmotic challenge. Water and glycerol permeability coefficients for both AQP12-overexpressing and -silenced cells were similar to wild-type cells.

It is worth noting that all the controls (silencing and overexpressing) depicted similar values to the native genotype for both AQP7 and AQP12 generated transfections (Figure 2.7), further validating our experimental model.

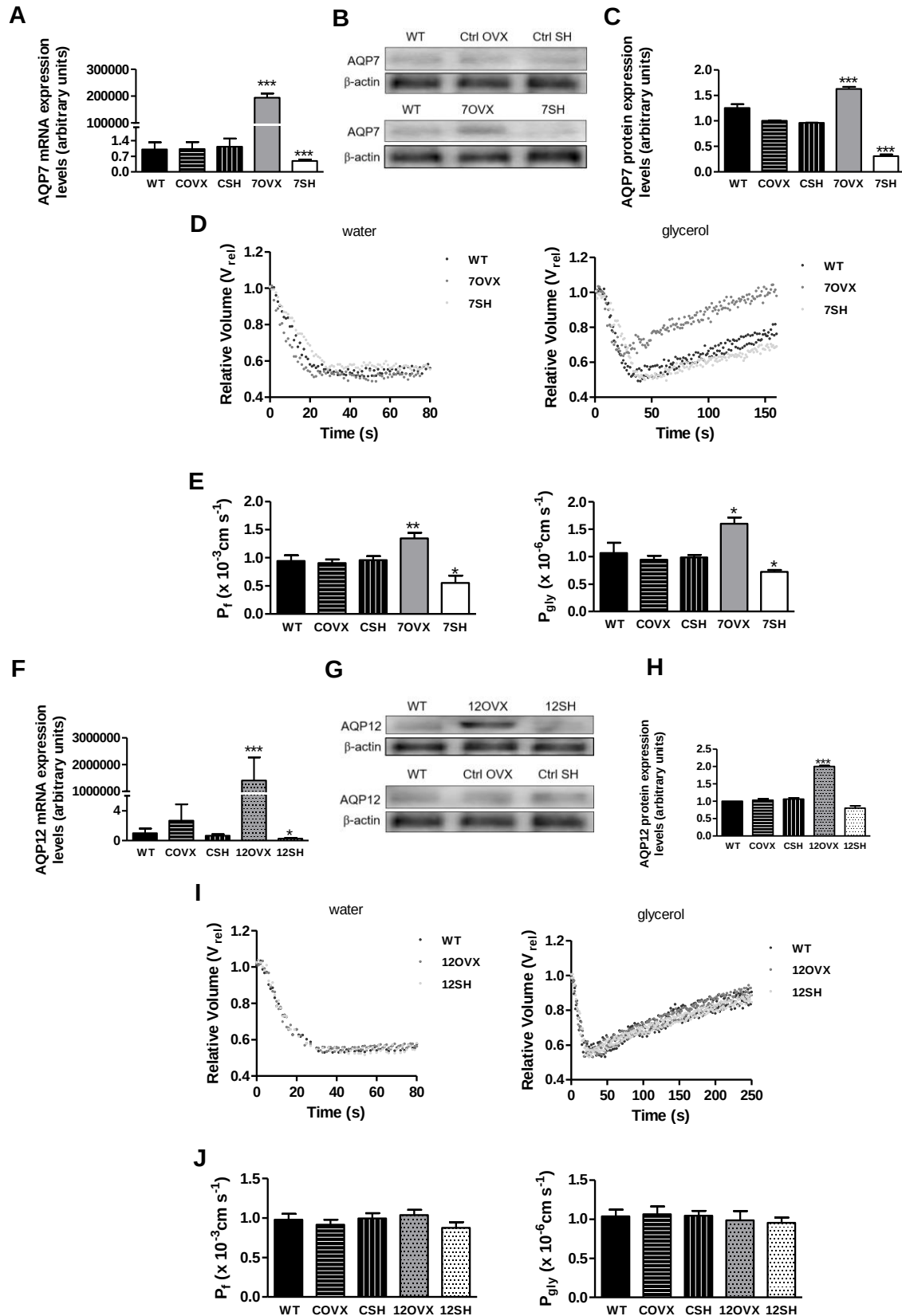


Figure 2.7. Validation of the model gain of function/loss of function. (A) mRNA expression levels of AQP7 normalized to 18S in cells obtained from WT, COVX, CSH, 7OVX and 7SH RIN-m5F cultures. (B) Representative blot of AQP7 protein in WT, COVX, CSH, 7OVX and 7SH RIN-m5F cultures. (C) Relative AQP7 expression, normalized to β-actin. (D) Time course

of cell volume changes caused by osmotic challenges with non-permeant and permeant solute (glycerol) in WT, 7OVX and 7SH cells; **(E)** Water (P_f) and glycerol permeability (P_{gly}). **(F)** mRNA expression levels of AQP12 normalized to 18S in cells obtained from WT, COVX, CSH, 12OVX and 12SH RIN-m5F cultures. **(G)** Representative blot of AQP12 protein in WT, COVX, CSH, 12OVX and 12SH RIN-m5F cultures. **(H)** Relative AQP12 expression, normalized to β -actin. **(I)** Time course of cell volume changes caused by osmotic challenges with non-permeant and permeant solute (glycerol) in WT, 12OVX and 12SH cells; **(J)** Water (P_f) and glycerol permeability (P_{gly}). Data represent a mean of three independent experiments $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *, transfected vs WT cells. WT, cells wild-type; COVX, overexpression control cells; CSH, silencing control cells; 7OVX, AQP7-overexpressing cells; 7SH, AQP7-silenced cells, 12OVX, AQP12-overexpressing cells; and, 12SH, AQP12-silenced cells.

Involvement of AQP7 and AQP12 in obesity-induced inflammation

To gain insights in AQP7 and AQP12 involvement in the development of a proinflammatory phenotype, RIN-m5F cells overexpressing and silenced for AQP7 (7OVX and 7SH) and AQP12 (12OVX and 12SH), were cultured with $\text{TNF}\alpha$ and with LPS.

In control conditions, 7OVX showed increased IL-6 ($(1.41 \pm 0.28) \times 10^{-7}$) and $\text{TNF}\alpha$ ($(8.57 \pm 1.39) \times 10^{-8}$) transcripts, while 7SH depicted similar values to control cells. In addition, 7OVX cells showed lower levels of $\text{TGF}\beta$ release ($162.26 \pm 7.90 \text{ pg mL}^{-1}$), contrasting with increased levels of $\text{TGF}\beta$ release in 7SH ($255.24 \pm 7.886 \text{ pg mL}^{-1}$), and seem to naturally release very low amounts of insulin ($1.55 \pm 0.05 \text{ mU L}^{-1}$) (Figure 2.8 and 2.9). These data suggests that a higher expression of AQP7 in RIN-m5F increases the inflammatory state and cell fails in performing its natural functions, such as insulin secretion [38].

Despite less is known about AQP12, this aquaporin isoform was described involved in exocrine pancreas physiological functions [31] which, together with its downregulation in pancreatitis, raised the question if this isoform might also be involved in the dysfunction of endocrine pancreas [32]. As depicted in Figure 2.8 and 2.9, our results show that cells with high levels of AQP12, as 12OVX, have naturally lower transcript levels of $\text{TNF}\alpha$ ($(3.96 \pm 0.75) \times 10^{-9}$) and release lower protein levels of IL-6 ($1.47 \pm 0.07 \text{ pg mL}^{-1}$), in contrast to 12SH that release higher levels of IL-6 ($2.07 \pm 0.07 \text{ pg mL}^{-1}$) comparing to control cells. Moreover, 12SH release lower levels of anti-inflammatory cytokines IL-4 ($3.43 \pm 0.07 \text{ pg mL}^{-1}$), IL-10 ($0.96 \pm 0.05 \text{ pg mL}^{-1}$) and $\text{TGF}\beta$ ($125.95 \pm 3.33 \text{ pg mL}^{-1}$). This data suggests a protective role for AQP12.

As previously described, WT cells developed a proinflammatory phenotype when cultured in the presence of TNF α and LPS (Figure 2.6), two molecules that activate distinct pathways but both triggering NF- κ B signaling and initiating the inflammatory process [39]. Figure 2.8 and 2.9 also depict the proinflammatory profile of AQP7 and AQP12-overexpressing and silenced cells as a response to TNF α and LPS treatment. After TNF α treatment, 7OVX showed increased IL-6 ((4.52 $\pm$ 0.92) $\times 10^{-6}$) and IL-1 β transcripts ((1.24 $\pm$ 0.09) $\times 10^{-6}$), values that were decreased in 7SH (IL-6, (2.65 $\pm$ 0.31) $\times 10^{-6}$; IL-1 β , (3.82 $\pm$ 0.65) $\times 10^{-8}$). Moreover, 7OVX revealed decreased levels of secreted IL-10 (0.91 $\pm$ 0.00 pg mL $^{-1}$) and insulin (1.00 $\pm$ 0.03 mU L $^{-1}$). 7SH also presented lower TNF α mRNA expression levels ((3.23 $\pm$ 0.66) $\times 10^{-5}$) and lower levels of IL-6 (1.87 $\pm$ 0.07 pg mL $^{-1}$), insulin (12.15 $\pm$ 1.70 mU L $^{-1}$) and C-peptide release (68.21 $\pm$ 3.37 pmol L $^{-1}$), comparing to control cells. Likewise, after LPS treatment 7OVX showed increased IL-1 β transcripts ((5.47 $\pm$ 3.90) $\times 10^{-7}$) while in 7SH those values were null. Contrary to the expected, 7OVX also presented higher levels of TGF β release (238.49 $\pm$ 15.95 pg mL $^{-1}$). LPS also impaired TNF α transcripts ((8.13 $\pm$ 3.42) $\times 10^{-7}$) and IL-6 protein release (0.30 $\pm$ 0.00 pg mL $^{-1}$) in 7SH cultures.

On the other hand, 12OVX cultured with TNF α resulted in decreased IL-6 (3.64 $\times 10^{-7}$ $\pm$ 7.62 $\times 10^{-8}$), IL-1 β (3.05 $\times 10^{-8}$ $\pm$ 3.96 $\times 10^{-9}$) and TNF α (4.52 $\times 10^{-6}$ $\pm$ 1.23 $\times 10^{-6}$) transcripts. IL-6 was also decreased at protein release level (1.33 $\pm$ 0.13 pg mL $^{-1}$) while IL-4 (7.033 $\pm$ 0.27 pg mL $^{-1}$) and TGF β (166.67 $\pm$ 6.90 pg mL $^{-1}$) were increased. Insulin release was increased (56.54 $\pm$ 1.80 mU L $^{-1}$) while C-peptide in media decreased (52.87 $\pm$ 0.62 pmol L $^{-1}$). Silenced AQP12 was reflected in lower TGF β (140.53 $\pm$ 4.20 pg mL $^{-1}$) and insulin (12.053 $\pm$ 1.39 mU L $^{-1}$) release. LPS induced decreased levels of IL-6 at both transcript ((2.83 $\pm$ 0.94) $\times 10^{-6}$) and protein release levels (0.34 $\pm$ 0.042 pg mL $^{-1}$) in 12OVX while IL-4 (4.97 $\pm$ 0.33 pg mL $^{-1}$) and TGF β (187.09 $\pm$ 12.46 pg mL $^{-1}$) release increased in these cells. 12SH showed augmented levels of IL-6 ((8.52 $\pm$ 2.55) $\times 10^{-6}$) and IL-1 β ((1.11 $\pm$ 0.08) $\times 10^{-7}$) transcripts and increased IL-1 β (1.67 $\pm$ 0.34 pg mL $^{-1}$) and TNF α (1.61 $\pm$ 0.27 pg mL $^{-1}$) protein release levels. TGF β release (255.24 $\pm$ 7.886 pg mL $^{-1}$) is impaired in 12SH cells.

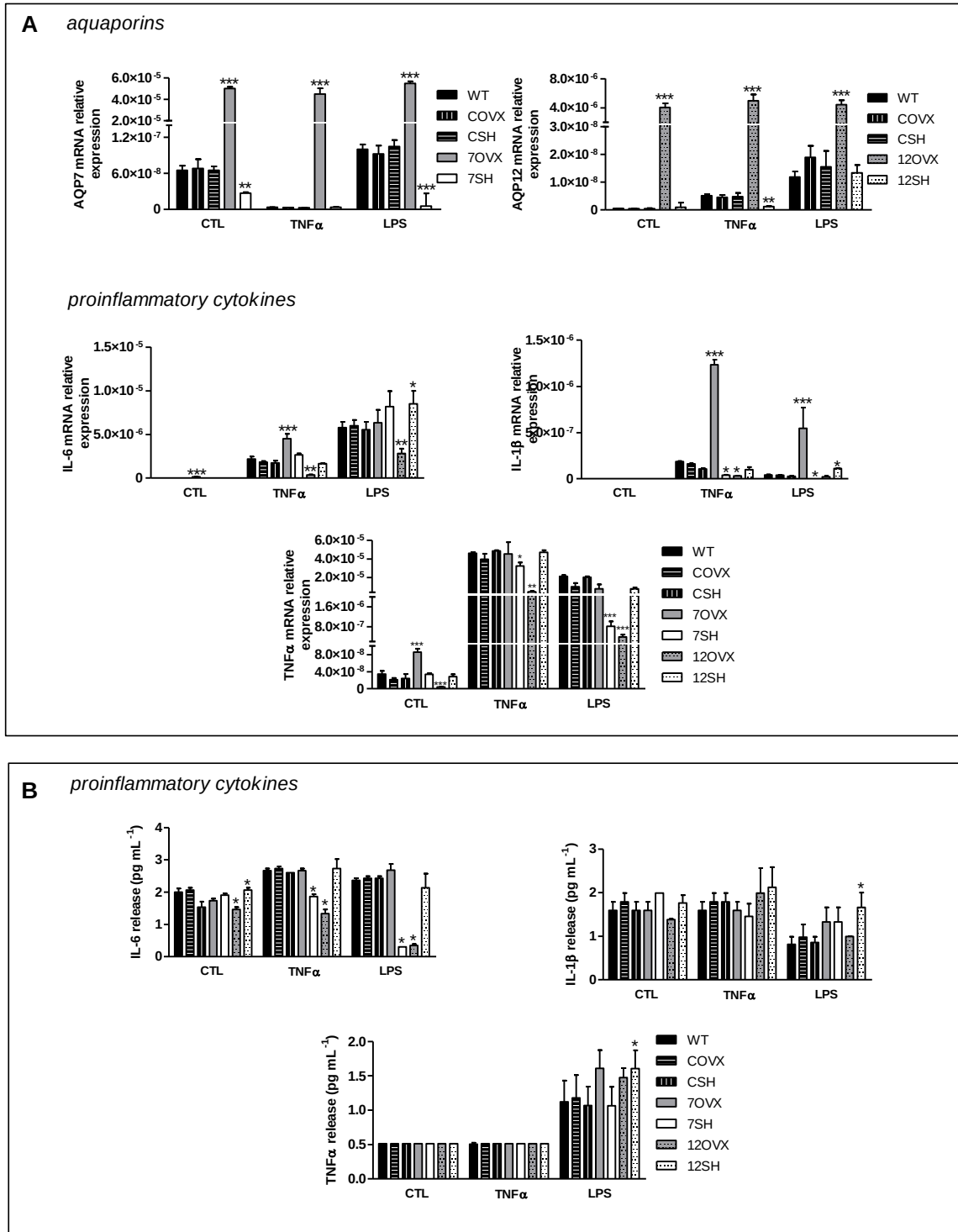


Figure 2.8. $TNF\alpha$ and LPS induce AQPs dysregulation and proinflammatory phenotype in RIN-m5F. (A) mRNA expression levels of AQP7 and proinflammatory cytokines IL-6, IL-1 β and $TNF\alpha$ normalized to 18S in cells silencing and overexpressing AQP7 and AQP12, non-treated and treated $TNF\alpha$ or LPS. **(B)** Protein release levels of proinflammatory cytokines (IL-6, IL-1 β and $TNF\alpha$) in supernatants from cells obtained in transfected RIN-m5F cells silencing and overexpressing AQP7 and AQP12, non-treated and treated with $TNF\alpha$ and LPS. Data represent a mean of three independent experiments $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *, transfected vs WT cells. WT, cells wild-type; COVX, overexpression control cells; CSH, silencing control cells; 7OVX, AQP7-overexpressing cells; and, 7SH, AQP7-silenced cells; 12OVX, AQP12-overexpressing cells; and, 12SH, AQP12-silenced cells.

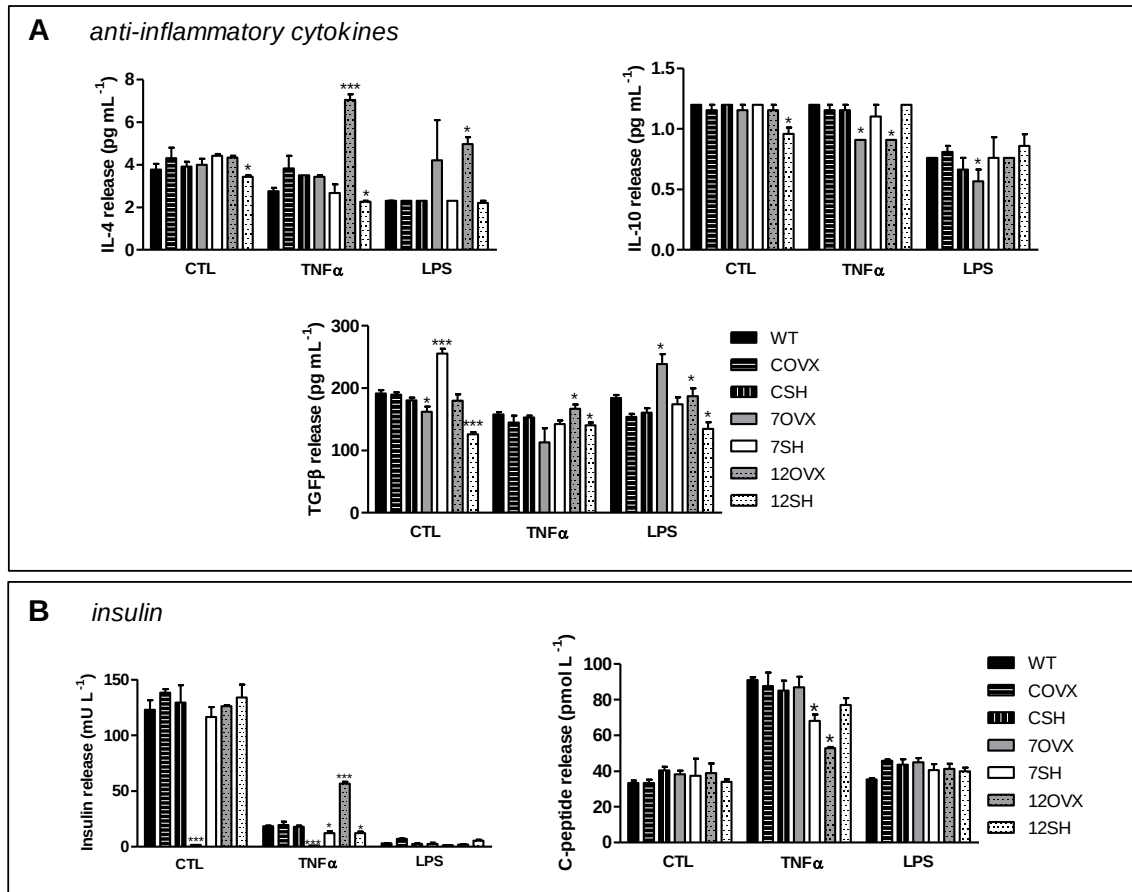


Figure 2.9. TNFα and LPS affects the physiological functions of in RIN-m5F. Protein release levels of anti-inflammatory cytokines (IL-4, IL-10 and TGFβ) (A) and Insulin and peptide-C release levels (B) in supernatants from cells obtained in transfected RIN-m5F cells silencing and overexpressing AQP7 and AQP12, non-treated and treated with TNFα and LPS. Data represent a mean of three independent experiments ± SEM. *, P<0.05; **, P<0.01; ***, P<0.001; *, transfected vs WT cells. WT, cells wild-type; COVX, overexpression control cells; CSH, silencing control cells; 7OVX, AQP7-overexpressing cells; and, 7SH, AQP7-silenced cells; 12OVX, AQP12-overexpressing cells; and, 12SH, AQP12-silenced cells.

AQP7 and AQP12 implications in cell proliferation, adhesion and migration

Subsequently, we evaluated the capability of proliferation, adhesion and migration in the generated cell lines. Wild type RIN-m5F cells showed a duplication time of 16 hours. Only AQP7-overexpressing cells showed significant differences in the proliferation rate when compared to controls, a 0.75-fold decrease (0.75 ± 0.008 -fold) in the duplication time (Figure 2.10A).

Interestingly, a higher adhesion ability of AQP7 and AQP12-overexpressing cells was observed when compared to AQPs-silenced and wild type pancreatic β -cells (Figure 2.10B).

Cell migration was evaluated using the scratch assay. RIN-m5F growing in monolayer showed a faster recover of the wounded area in cells overexpressing AQP7 (2.05 ± 0.17 -fold) comparing to control cells, and cells silenced for AQP7 were around 40% slower (0.61 ± 0.08 -fold). AQP12-overexpressing and -silenced cells showed wound closure features similar to control cells (Figure 2.10C).

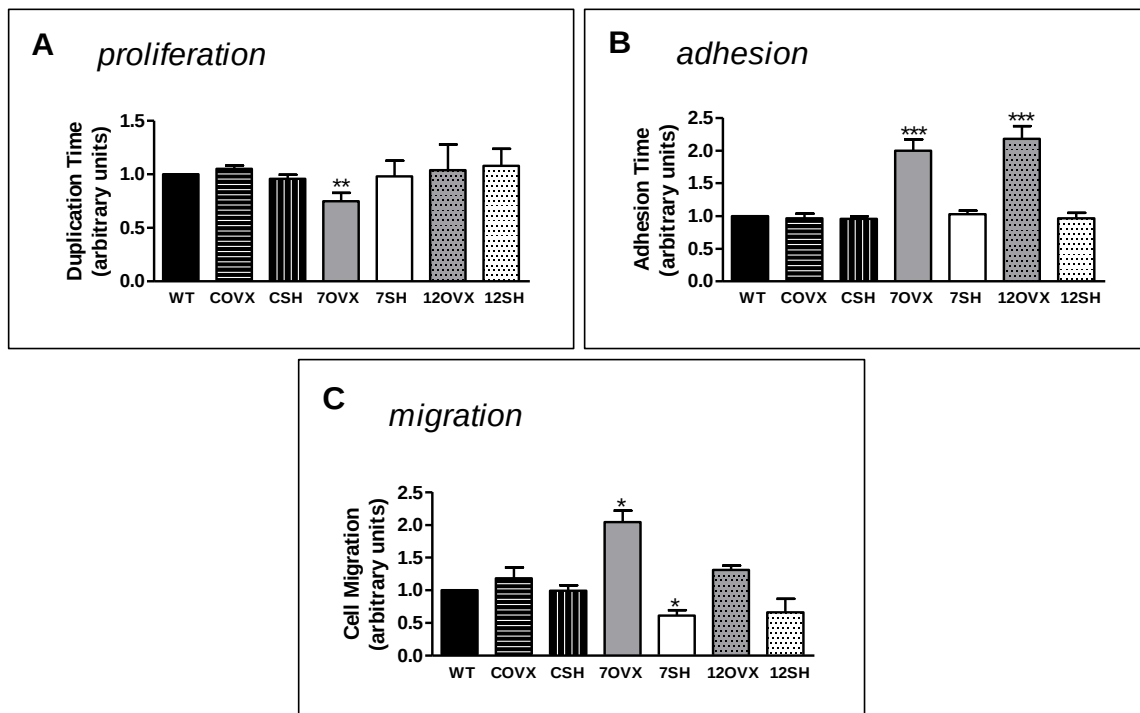


Figure 2.10. AQP7 and AQP12 have implications in proliferation, adhesion and migration. Relative duplication time (A), adhesion time (B) and cell migration (C) of transfected RIN-m5F cells silencing and overexpressing AQP7 and AQP12. Data represent a mean of three independent experiments $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *, transfected vs WT cells. WT, cells wild-type; COVX, overexpression control cells; CSH, silencing control cells; 7OVX, AQP7-overexpressing cells; 7SH, AQP7-silenced cells, 12OVX, AQP12-overexpressing cells; and, 12SH, AQP12-silenced cells.

Discussion

Aquaporins have a preponderant role in maintaining water homeostasis and energy balance through the body. Recently, aquaglyceroporins were shown to have a close involvement in obesity and metabolic-related pathologies, such as type-2 diabetes and pancreatitis, being obesity the main risk factor for the development of such pathologies.

Lately, using molecular approaches, AQP7 was shown to be highly expressed in the rat pancreatic β -cells of the islets of Langerhans at both transcript and protein levels [33]. Such aquaporin isoform, mapped to chromosome 9p13.3 in human, was firstly cloned from adipose tissue in 1997 (originally AQPap) and in this tissue it plays a crucial role in the control of TAG accumulation and systemic glucose homeostasis [40,41,42]. Aqp7-KO mice exhibits adult-onset obesity, impaired insulin secretion and insulin resistance [30]. Here, we used a pancreatic β -cell line, RIN-m5F, that expresses AQP7 and AQP12 to clarify the contribution these isoforms in the development of obesity-associated inflammation in endocrine pancreas. Our data show that AQP7 has highly expressed in these cells, while the intracellular isoform AQP12 is expressed in much lower amounts (Figure 2.5A, B and C). Therefore, the measured water and glycerol permeability are mainly due to AQP7 contribution (Figure 2.5D and E).

Studies carried out in the last decades identified inflammation as the most important link between obesity and type-2 diabetes, and the unifying mechanism capable of affecting both the action and production of insulin [43]. The development of obesity triggers an inflammatory state in tissues involved in metabolism and energy homeostasis. Moreover, during the progression of obesity and insulin resistance, the pancreatic islets are also affected by inflammation, contributing to β -cell failure and leading to the onset of T2D [44].

This study was designed to understand the involvement of AQP7 and AQP12 in endocrine pancreas inflammation as a consequence of obesity and type-2 diabetes. In this sense, we achieved a proinflammatory phenotype through activation of TNF α receptor, one of the pathway that leads to the activation of NF- κ B, resulting in the upregulation of proinflammatory genes and downregulation of anti-inflammatory genes, impaired insulin secretion and ultimately β -cell failure and death [45,46]. Here, we show that excess TNF α downregulates AQP7 while AQP12 is upregulated, and proinflammatory cytokines are augmented at both transcript (IL-6, IL-1 β and TNF α) and protein release levels (IL-6) and anti-inflammatory cytokine release is impaired (IL-4 and TGF β) (Figure 2.6A and B). In addition, insulin secretion was affected in parallel with increased levels of C-peptide in supernatants of cells treated with TNF α (Figure 2.6B).

By the means of transfecting cells to overexpress and silence AQP7 it was possible to gain insight on cells individual response when osmotic and solute gradients were applied and ultimately assess the values of P_f and P_{gly} with minimal alterations to cells

physiological status. Firstly, we were able to determine that AQP7 expressed in RIN-m5F rat pancreatic β -cells is functional and behaves as a water/glycerol channel, as previously described [25]. AQP7 activity was closely connected to their transcript and protein levels, being augmented in AQP7-overexpressing cells and greatly impaired in AQP7-silenced cells (Figure 2.7A-D). Moreover, the calculated permeability values are within the range published for other aquaglyceroporin-expressing cells [20,36]. A similar experimental strategy has been previously applied by our group to demonstrate the role of AQP7 as water/glycerol channel in adipocytes [35]. In RIN-m5F, although AQP12 mRNA and protein expression (basal expression, overexpression and silenced expression) were correlated, the gain of function/loss of function strategy was not successful to evaluate AQP12 function and selectivity, since the permeability assay was performed in whole cells and thus only discriminates plasma membrane channels. Further experiments with isolated subcellular membrane fractions are necessary to characterize AQP12 functional behavior in pancreatic cells.

Aquaporins were also shown to be implicated in several biological processes due to their function as water and glycerol channels. It was already demonstrated that AQP7 contributes for pancreatic β -cell proliferation by allowing fast cell volume regulation during cell division [25,47]. Our results confirm that AQP7 expression has implications in cell proliferative mechanisms (Figure 2.10A). Higher AQP7 expression was related to higher proliferative rates. Additionally, a clear higher adhesion ability was also observed in cells with higher AQP7 and AQP12 expression. Other aquaporins, AQP0 and AQP4, have also been reported important in cell-cell adhesion [48,49,50]. Furthermore, AQP7 expression showed to favor β -cell migration.

As previously described, WT cells developed a proinflammatory phenotype when cultured in the presence of TNF α and LPS (Figure 2.6), two molecules that activate distinct pathways but both triggering NF- κ B signaling and initiating the inflammatory process [39]. Interestingly, the inflammatory status of AQP7 and AQP12-overexpressing and silenced cells cultured in control conditions was naturally affected. In Figure 2.8 and 2.9, we observe few inflammatory features in 7OVX and 12SH comparing to WT, when cultured in control media. Additionally, when cells were exposed to TNF α and LPS, proinflammatory features were exacerbated or smoothened according to each transfection, indicating the contribution of these two isoforms to the inflammation in pancreas.

AQP7 was previously shown to be downregulated by TNF α in 3T3-L1 adipocytes [51] while LPS has been linked with impaired insulin secretion [37]. In WT RIN-m5F monoculture treated with TNF α , we also observed lower levels of AQP7 transcript while AQP7 was increased with LPS treatment. Although 7SH seem to be less affected by the proinflammatory effects of TNF α , these cells secrete less insulin, possibly due to the defective glycerol transport. On the other hand, 7OVX revealed a profile of cell failure where insulin secretion was nearly absent. These results suggest that higher levels of AQP7 in the presence of TNF α might be a result of chronic obesity, where cells with higher AQP7 expression try to compensate hyperglycemia by producing elevated levels of insulin, but the system fails; on the other hand, low levels of AQP7 might mime an acute stress, such as T2D, where cells still did not failed but are not capable to release the amount of insulin needed. LPS treatment seems to have weaker effect in AQP7 function in RIN-m5F since insulin secretion was not compromised. We conclude that activation of TNF α receptor might compromise AQP7 function in endocrine pancreas in a more effective manner than activation of TLRs.

AQP12 was found intracellularly expressed in pancreatic acinar cells of the exocrine pancreas, and AQP12-null mice showed more severe pathological damage than WT mice when pancreatitis was induced [31,32]. Accordingly, our findings suggest that the expression of AQP12 might have a protective role. Experiments done in 12OVX and 12SH cells treated with TNF α and LPS, demonstrated that AQP12 expression is correlated with lower levels of proinflammatory cytokines, higher levels of anti-inflammatory cytokines and improvement of insulin secretion (Figure 2.8 and 2.9).

Conclusions

This work demonstrates that AQP7 and AQP12 are involved in pancreatic β -cells proliferation, adhesion and migration. Moreover, while AQP7 is closely related to insulin secretion, due to its role as a glycerol channel, AQP12 is unequivocally entangled in the inflammatory status of the pancreatic cell. Targeting AQP7 and AQP12 may open new perspectives for anti-diabetic drug development.

Material and Methods

Cells culture and treatments

Rattus norvegicus pancreatic β -cell line (RIN-m5F) was purchased from ATCC (ATCC CRL 11605™). RIN-m5F cells were cultured at 37°C, 5% CO₂ atmosphere in RPMI 1640 medium (Biowest, Riverside, MO, US) containing 10% fetal bovine serum (Hyclone). Cells were used for experiments at a confluency of 70-80%. Treatments with TNF α (10 ng/mL, Sigma) and LPS (100ng/mL, Sigma) TNF α were performed in parallel with cells growing with non-treated media for 24 hours and 4 hours, respectively.

Transfection of RIN-m5F cell line

AQP7 and AQP12 overexpression was performed in RIN-m5F cell line with commercial expression vectors (Genecopeia, Rockville, MD, US) encoding for the respective rat aquaporin ORF. For each AQP knock-down, four different short hairpin RNA (shRNA)-containing expression vectors were used according to the manufacturers 'protocol. Controls of overexpression and silencing were also produced in parallel. For cell transfection, cells were seeded with an inoculum of 40 000 cells/cm² in 6-well plates and the instructions recommended by Genecopeia were followed. Five days post-transfection cells were trypsinized and when cells reached confluence of 70-80% selection with 1 μ g/mL puromycin (Sigma, Kawasaki, Kanagawa, Japão) was initiated. Transfections were validated by qPCR, Immunoblotting and functional assays.

RNA extraction and quantitative PCR

Total RNA was isolated from samples with 1 million of cells using Trizol® (Life Technologies), and extraction was performed according to manufacturer instructions. cDNA was synthesized from 1 μ g total RNA and the RT-PCR was carried out using a SuperScript III kit (Invitrogen). Real-time PCR reactions were carried out using a CFX96 Real-Time System C1000 (BioRad), the TaqMan Universal PCR Master Mix (Applied Biosystems) and the following specific TaqMan pre-designed gene expression primers and probes (Applied Biosystems): AQP7 (FWD 5'-GGCTTCGTGGATGAGGT ATTTG-3', REV 5'-ACAGTCCAGCACTTCAAGGGAC-3' and Probe 5'-FAM-AGCTGTG TATCTTCGCCATCACG-TAMRA-3'), AQP12 (FWD 5'-TCCACTGTTCTGGGAACACCTT-3', REV 5'-TCTGCCGGTAGAACAGATTTCT CT-3' and Probe 5'-FAM-ATCCTGGCTGTCCTACTCCATCAGGGC-TAMRA-3') and 18S (Rn03928990_g1). For IL-6 (FWD 5'- GGATACCACCCACAACAGACC-3' and REV 5'-AGTGCATCATCGCTGTTTCATACA-3'), IL-1 β (FWD 5'-

TGTCTGACCCATGTGAGCTGA-3' and REV 5'-TCGTTGCTTGTCTCTCCTTGTACA-3') and TNF α (FWD 5'-TCTTCTCATTCTGCTCGTGG-3' and REV 5'-GTCTGGGCCATGGAAGTAT-3') pre-designed primers were used (Metabion, Germany) and qPCR was performed using SYBR® Green Master Mix (Life Technologies) according to manufacturer instructions. The Ct method ($2^{-\Delta\Delta C_t}$) was used for relative quantification of target genes expression after normalization with the 18S reference gene [52,53].

Immunoblotting

Proteins were separated by SDS-PAGE before transfer. After blocking, the following specific primary antibodies were used: anti-AQP7 (Santa Cruz Biotechnology) and anti-AQP12 (ThermoFisher) antibodies (1:200 and 1:100, respectively), and anti- β -actin (1:1000; Sigma-Aldrich). Secondary anti-rabbit and anti-mouse (1:10000; Jackson Immuno Research) HRP-linked antibodies were used for ECL-mediated (Amersham Biosciences) detection. Band intensity was measured using the ImageJ software (<https://imagej.nih.gov>).

Permeability assays

Water (Pf) and glycerol (Pgly) permeability were measured in individual adherent cells on coverslips, as previously described [54,55]. Briefly, cells were loaded with 5 mM calcein acetoxymethyl ester (calcein-AM; Sigma-Aldrich) for 30 min at 37°C in 5% CO₂. The coverslips with the adherent cells were mounted a perfusion chamber (Warner Instruments, Hamden, USA) on the stage of a Zeiss Axiovert 200 inverted microscope. Fluorescence was excited at wavelength 495/10nm and the emission fluorescence collected with a 535/25 nm bandpass filter coupled with a 515nm dichroic beam splitter. Images were captured using a $\times 63$ magnification and a digital camera (CoolSNAP EZ, Photometrics, USA) and recorded by the Metafluor Software (Molecular Devices, USA). Cells were perfused with 300 mM HEPES buffer (135 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D-Glucose, 5 mM HEPES, pH 7.4, 300 mOsM) for 60 s, after which 300 mM mannitol (for water permeability) or 300 mM glycerol (for glycerol permeability) was added to the buffer to achieve an external osmolarity of 600 mOsM. Cell volume (V) was measured at selected time points from 2D images obtained during the permeability assays to evaluate the initial volume (prior to the osmotic challenge V₀) and the final equilibrium volume [37]. Pf and Pgly coefficients were evaluated from the measured time dependent volume changes, $V_{rel} = V/V_0$, obtained by adding mannitol (Pf) or glycerol (Pgly) to the external media, using the model equations described by Madeira et al. [35] and the Berkeley Madonna software (<http://www.berkeleymadonna.com>).

Proliferation and adhesion assays

Proliferation assays were conducted using crystal violet staining. For proliferation assay, cells were seeded with an inoculum of 25 000 cells/cm² in 96-well plates. After adhering, cells were washed with PBS to remove non-adherent cells. Adherent cells were fixed with 96% ethanol for 10 minutes and then stained with 0.1% crystal violet in 10% ethanol for 5 minutes at room temperature. After staining, the extracellular dye was removed by rinsing thoroughly the cell monolayer with tap water. The remaining cell-attached dye was dissolved in 200µL of 96% ethanol in 1% acetic acid and absorbance was measured 595nm. Four time points were measured in total.

Migration assays

RIN-m5F cells were seeded in 12-well plates with an inoculum of 25 000 cells/cm² and were allowed to grow till 70-80% confluence. Then, a scratch was done in the cell monolayer using a sterile 10 µL tip. After washing with PBS to remove cell debris, cells were then incubated at 37 °C in a 5% CO₂ incubator, and images of the wound were captured at intervals of 6 h. The distance of the wound was measured under a light microscope and analyzed using the software ImageJ (<https://imagej.net>). Wound closure was normalized to original wound area at time 0 and to the control.

ELISA assays

Cell-free supernatants were collected and clarified by centrifugation. IL-6, IL-1β, TNFα, IL4, IL-10 and TGFβ were quantified by ELISA following the kit supplier' protocol instructions (R&D Systems). Insulin and C-peptide was quantified following the kit supplier' protocol instructions (Roche) and values were measured in a Cobas 6000 (Roche)using the electrochemiluminescence method.

Statistical analysis

All the experiments were performed in three biological and at least two technical triplicates. Results were expressed as mean ± SD of n individual experiments. Statistical analysis between groups was performed by two-way ANOVA and non-parametric Mann–Whitney test. P values <0.05 were considered statistically significant. Statistical analyses were performed using the Graph Prism software (GraphPad Software).

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2.3. Involvement of aquaporin-3 in NLRP3-inflammasome priming and activation in THP-1 macrophages-like cells

This subchapter is adapted from following manuscript:

Inês Vieira da Silva, Carlos Cardoso, Helios Martinez-Banaclocha, Angela Casini, Pablo Pelegrin and Graça Soveral. Aquaporin-3 is involved in NLRP3-inflammasome priming and activation in THP-1 macrophages-like cells. (*in preparation*)

Abstract

Inflammasomes are large immune multiprotein complexes that tightly regulate the production of the pro-inflammatory cytokines, being dependent on cell regulatory volume mechanisms. Aquaporins (AQPs) are protein channels that facilitate the transport of water and glycerol (aquaglyceroporins) through membranes, essential for cell volume regulation. Variations in cell osmolarity and consequent cell volume adaptation precede NLRP3 inflammasome activation and interestingly, AQP blockage was shown to limit IL-1 β release from NLRP3-activated macrophages. However, the role of AQPs in inflammation is still unclear. Human peripheral blood monocytes (HPBMs) and THP-1 macrophages cultured under stimuli for cell priming and inflammasome activation are valuable models to study inflammation. Here, AQPs expression was evaluated in HPBMs with healthy or pro-inflammatory phenotype. Then, THP-1 macrophages were used to investigate AQPs as players and potential new targets in inflammation. We found that aquaglyceroporins AQP9 and AQP3 are the most representative isoforms in primary monocytes (HPBMs) and THP-1 cells, respectively, being both upregulated by LPS-priming. PMA-differentiated macrophages-like cells and LPS-primed macrophages-like cells showed similar basal water and glycerol permeability, being the latter reduced by Auphen, an AQP3 inhibitor. AQP3 inhibition partially blocked LPS-priming and decreased mRNA expression and protein release levels of IL-6 and IL-1 β , suggesting AQP3 involvement in macrophage priming by Toll-like receptor 4 engagement. NLRP3 inflammasome priming and activation was also blocked when AQP3 was inhibited, decreasing mRNA expression and protein release of IL-1 β after NLRP3 activation with nigericin and ATP. Moreover, challenging LPS-primed cells with hyperosmotic solutions of glycerol increased IL-1 β release through Caspase-1 activation. Altogether these data evidence AQPs as candidate players in the setting of the inflammatory response.

Keywords: aquaporins; inflammasome; inflammation; interleukin-1; macrophages; water and glycerol permeability

Introduction

Inflammasomes are large immune multiprotein complexes that tightly regulate the production of the pro-inflammatory cytokines interleukin IL-1 β and IL-18 [28, 29]. Several studies have demonstrated that NLRP3 inflammasome can be involved in metabolic homeostasis dysregulation, such as type-2 diabetes, fatty liver disease and gout [1,2,3,4,5]. The NLRP3 inflammasome is a major component of the innate immune system and is upregulated in response to different pathogen-associated molecule patterns (PAMPs) [6], or danger-associated molecule patterns (DAMPs) [7,8] that are signals of stress or injury.

The NLRP3 inflammasome is comprised by a cytosolic sensor NOD-like receptor, the protein NLRP3; an adaptor protein, apoptosis-associated speck-like protein containing a caspase activating recruitment domain (ASC); and an effector molecule, the cysteine protease pro-caspase-1. Two signals finely regulate the activation of NLRP3 inflammasome. The first signal, cell priming, triggers the nuclear factor NF- κ B enhancing the expression of the inflammasome components [9]. The synthesis of IL-6, pro-IL-1 β and pro-IL-18 is initiated by the ligation of a pathogen recognition receptor (PRR) such as the Toll-like receptor 4 (TLR4) with its respective ligand, e.g. lipopolysaccharide (LPS). The second signal, the inflammasome activation, promotes the assembly of the inflammasome components. During activation pro-caspase-1 is recruited, generally facilitated by ASC, and processes pro-IL-1 β and pro-IL-18 to their mature and active forms, IL-1 β and IL-18. Caspase-1 also cleaves the cytosolic protein gasdermin D to promote plasma membrane pore formation, driven into the release of cytokines and cell death by pyroptosis [10]. The exact mechanisms enrolled with the activation of inflammasomes are not fully understood, however, evidences involving the generation reactive oxygen species (ROS), lysosomal damage, and potassium (K⁺) efflux have been reported [11,12,13,14]. More recently, cell re-swelling has also been pointed as an event that precedes macrophage activation and consequent IL-1 β secretion [13,15,16]. Figure 2.11 depicts a schematic model showing the mechanism of inflammasome priming and activation in macrophages where AQPs are pointed to play a role.

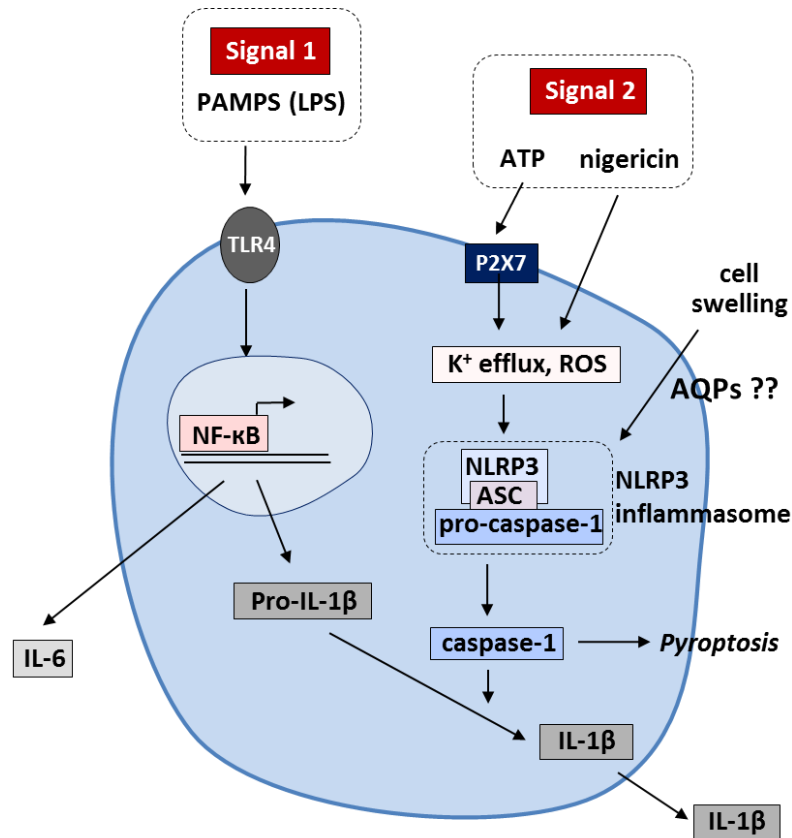


Figure 2.11. NLRP3-inflammasome priming and activation in macrophages. Two signals regulate the activation of NLRP3 inflammasome. During signal 1 (cell priming), TLR activation triggers the nuclear factor NF-κB enhancing the expression and synthesis of proinflammatory cytokines (IL-6, pro-IL-1β and pro-IL-18). The signal 2 (inflammasome activation) promotes the assembly of the inflammasome components. Pro-caspase-1 is recruited and activated and thus, it is able to process pro-IL-1β and pro-IL-18 to their mature and active forms, IL-1β and IL-18, respectively. Caspase-1 also promotes plasma membrane pore formation, driven into the release of cytokines and cell death by pyroptosis.

The regulatory volume mechanism described for NLRP3-inflammasome activation is not exclusive for macrophages; in fact, the ability to adjust and adapt its volume is vital for survival and proper functioning to any animal cell. Subsequently, proteins involved in the regulation of cell volume may have a key role in the onset of inflammation. Recently, aquaporins were proposed to be involved in the setting of inflammasome activation [13,15,16,17,18].

The aquaporins (AQP) are a family of highly conserved transmembrane protein channels that facilitate the transport of water and small uncharged solutes over the plasma membrane [19,20]. Since the first AQP was identified by Peter Agre in 1988 [21], 13 human AQP, AQP0-AQP12 have been described and they were divided into three subfamilies based on their pore-selectivity and primary structure. Orthodox AQPs

are considered strict water channels (AQP0, 1, 2, 4, 5, 6, 8, although AQP6 and AQP8 also transport anions [22] and ammonia [23]; aquaglyceroporins (AQP3, 7, 9, 10) allow the transport of water and small uncharged molecules, such as glycerol and urea; and, unorthodox AQPs (AQP11, 12) are subcellular isoforms whose selectivity and function still needs clarification [24,25]. Additionally, a few isoforms also facilitate hydrogen peroxide membrane permeation and are called peroxiporins [26,27,28,29]. Aquaporins are important for numerous cellular functions, such as epithelial fluid transport, cell proliferation, brain water homeostasis and adipocyte metabolism and are considered potential drug targets for human diseases [30]. Furthermore, by interacting with the cytoskeleton and signaling cascades, AQPs can influence cell morphology, volume, movement and migration [30,31]. The involvement of AQPs in the development of inflammatory processes has been extensively considered since several isoforms are expressed in cells of both innate and adaptive immunity while their dysregulation was demonstrated in various human diseases [32,33]. AQPs seem to be involved in the phagocytosis, and in immune cells activation and migration [18,34,35,36,37]. Furthermore, AQPs blockage or silencing reduces IL-1 β release from macrophages activated through a variety of NLRP3 stimuli [16]. Thus, inhibition of AQP in macrophages specifically during the regulatory volume decrease process is sufficient to limit IL-1 β release through the NLRP3 inflammasome axis [13]. These findings unveil a new function of AQPs in the inflammatory process and suggest a novel therapeutic target for anti-inflammatory therapy.

AQPs are expressed in human immune cells both in the innate and the adaptive immune system. To date, AQP1 and AQP9 were detected in human blood leukocytes and it are upregulated after intravenous LPS stimulation in humans [38,39]. AQP9 is also augmented in the systemic inflammatory response syndrome [40] and infective endocarditis [41]. Activated B and T lymphocytes were reported to express AQP1, AQP3 and AQP5, immature dendritic cells express AQP3 and AQP5, and their expression was related to activation and proliferation of these immune cells [32]. In leukocytes AQP9 was found to preferentially localize to the cell edges possibly facilitating motility, lamellipodium extension and stabilization, and cell volume changes enabling these cells to move toward chemoattractants [42]. AQP9 upregulation was also detected in human primary blood-derived macrophages and neutrophils when stimulated with LPS at both transcript and protein levels [43]. Studies in dendritic cells of AQP7-KO mice showed that AQP7 is involved in the process of chemokine-

dependent migration and antigen uptake and processing [44]. Moreover, the peroxiporin activity of AQP3 was reported to have a crucial role during T-cell migration in a chemokine gradient [45]. Altogether, these data strongly suggest that AQPs play a key role in the immune response.

In this study, we used human peripheral blood monocytes (HPBM) and THP-1 macrophages cultured under stimuli for cell priming and inflammasome activation to trigger a proinflammatory phenotype. We investigated the expression of aquaporins along proinflammatory markers comparing to the healthy phenotype. Subsequently, we evaluated the contribution of AQPs to cell volume changes in resting, non-primed and LPS-primed THP-1 cells by measuring cell membrane water and glycerol permeability and using Auphen as an aquaglyceroporin inhibitor. In order to determine the contribution of AQPs to the subsets of inflammation we: 1) performed the cell priming in the absence and presence of Auphen and evaluate IL-6 and IL-1 β expression at different levels including protein release, and parallelly evaluate LDH leakage to infer Auphen toxicity and cell death by pyroptosis; and, 2) activated the inflammasome through hyperosmotic solution of glycerol, nigericin and ATP in the absence and presence of Auphen and evaluate IL-1 β expression and cell death by pyroptosis. Moreover, knowing that in addition to the cell volume regulation cytosolic K⁺ depletion stimulates NLRP3 inflammasome activation, we measured internal K⁺. Finally, the effect of AQPs inhibition in IL-1 β release was also evaluated.

Results

AQP9 is the most expressed isoform in HPBM and is upregulated in activated macrophages

Enhanced cell volume and surface area, and cell shape and protrusion fluctuations in macrophages are processes that, being driven by fluxes of water across cell membrane, have been attributed to aquaporins. AQP9, as the predominant AQP isoform in human macrophages, has been pointed to be the responsible for those water movements [36]. In the inflammatory process, where inflammasome activation leads to a cell response against a variety of danger signals perception by secreting proinflammatory interleukins, rapid volume changes precede IL-1 β and IL-18 release, and in fact, a few authors have suggested that AQPs, as water channels, might be

involved in that event [13]. Our screen through AQP isoforms is in agreement with the previous finding, being AQP9 the most representative isoform (142.130 ± 65.738) in human monocytes and LPS-activated macrophages (Figure 2.12A). In addition, AQP3 (1.028 ± 0.465), AQP6 (0.005 ± 0.002), AQP8 (0.064 ± 0.059), AQP11 (0.195 ± 0.200) and AQP12 (0.131 ± 0.171) were also detected in these cells but in lower amounts (Figure 2.12A). Interestingly, inducing the activated-macrophage phenotype by priming cells with LPS (signal 1 for inflammasome activation) to trigger NF- κ B signaling and significantly upregulating mRNA expression levels of IL-6 (159.165 ± 13.105 vs 0.285 ± 0.229 in resting; $p < 0.001$), IL-1 β (668.393 ± 97.394 vs 70.522 ± 40.232 in resting; $p < 0.01$) and TNF α (101.476 ± 34.016 vs 3.505 ± 0.854 in resting; $p < 0.01$) (Figure 2.12B), also induced a 8.2-fold in AQP6 (0.045 ± 0.039 ; $p < 0.01$) and a 4.7-fold increase of AQP9 levels (661.587 ± 267.589 ; $p < 0.01$) (Figure 2.12A). This finding suggests that AQP9 plays a part in the inflammatory response mechanism and its role in inflammatory settings should be clarified.

The means by which aquaporins function can be studied and understood in this model showed to be challenging. The general blocker of aquaporin function, HgCl₂, is highly toxic and thus not usable when studying the signal 2 for inflammasome activation, and the transfection success for gene silence in human monocyte primary cells is very low. To overcome these gaps, we used an immortalized human monocyte cell line, THP-1. Aquaporin screening in THP-1 line was performed in resting monocyte-like cells, PMA-differentiated non-activated macrophage-like cells and PMA-differentiated LPS-activated macrophage-like cells, from now on referred as resting, non-activated and LPS-activated cells. In all the defined conditions, we detected expression of AQP1, AQP3, AQP5, AQP7, AQP8, AQP9 and AQP11, while AQP5 was only detected in resting cells (Figure 2.12C). AQP3 (0.306 ± 0.015) is the most expressed aquaporin in THP-1 cells and, even with a weaker response to LPS-activation compared to AQP9 behavior in HPBM, it is upregulated (0.446 ± 0.032 ; $p < 0.05$) by a 100 ng/mL for 24h treatment together with AQP9 ($9.403 \times 10^{-3} \pm 1.265 \times 10^{-3}$ vs non-activated cells $5.282 \times 10^{-3} \pm 0.442 \times 10^{-3}$; $p < 0.05$) while AQP7 ($0.155 \times 10^{-3} \pm 3.96 \times 10^{-5}$ vs non-activated cells $0.213 \times 10^{-3} \pm 0.065 \times 10^{-3}$; $p < 0.01$) and AQP8 ($1.172 \times 10^{-3} \pm 7.64 \times 10^{-6}$ vs non-activated cells $2.661 \times 10^{-3} \pm 0.310 \times 10^{-3}$; $p < 0.05$) seem to be downregulated (Figure 2.12C).

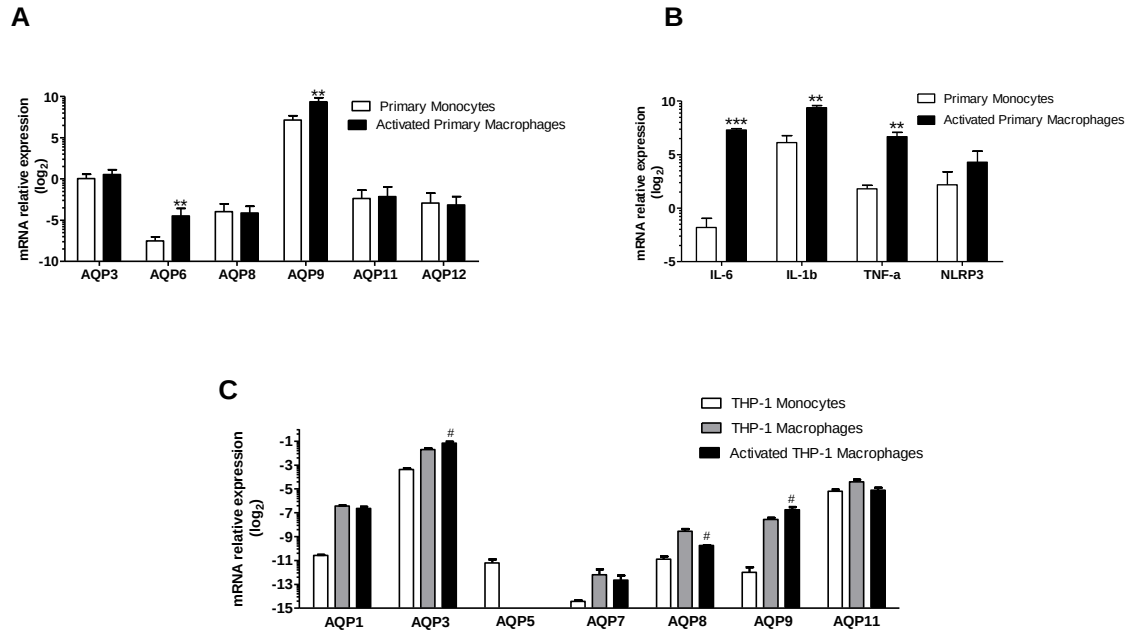


Figure 2.12. Aquaporins and inflammatory markers gene expression and their modulation by LPS in human peripheral blood monocytes (HPBMs) and THP-1 cell line. (A) mRNA expression of AQP3, 6, 8, 9, 11 and 12 in primary monocytes and macrophages (HPBMs). (B) mRNA expression of IL-6, IL-1 β , TNF α and NLRP3 in primary monocytes and macrophages (HPBMs). (C) mRNA expression of AQP1, 3, 5, 7, 8, 9 and 11 in THP-1 monocytes, macrophages and LPS-activated macrophages. Values are relative to HPRT-1. Data represent mean $\pm$ SD of three independent experiments. **, $P < 0.01$, ***, $P < 0.001$; *, primary macrophages vs primary monocytes; #, $P < 0.05$; #, activated THP-1 macrophages vs THP-1 macrophages

AQP3 is an active glycerol channel in THP-1 cells

It is already known that AQPs are expressed in macrophages and that they might be responsible for the rapid volume changes needed for inflammasome activation during an inflammatory response [13], however their activity has never been measured in these cells. Since we found AQP3 as the representative isoform in THP-1 cells, we proceeded to validate its role as a water and glycerol channel, taking advantage of the potent AQP3-specific inhibitor Auphen [46].

Two distinct methods were used to evaluate water (P_f) and glycerol permeability (P_{gly}) and Auphen inhibitory effect in THP-1 cell line. By stopped-flow light scattering we are able to measure membrane permeabilities in resting cells, as close as possible to their physiological condition. For this purpose, resting THP-1 cells incubated in isotonic phosphate buffered saline (PBS) solution were challenged with hyperosmotic

mannitol (impermeant solute, inducing water efflux and cell shrinkage) or glycerol (permeant solute, cells shrink due to the hyper-osmotic gradient and re-swell due to glycerol entrance) solutions. From the rate of cell volume changes (shrinkage and re-swelling) after imposed osmotic gradients, we calculated the membrane permeability to water ($P_f = (5.259 \pm 0.693) \times 10^{-4}$) and glycerol ($P_{gly} = (0.046 \pm 0.006) \times 10^{-4}$) (Figure 2.13A) and the activation energy for water and glycerol transport ($E_a = 9.106 \pm 0.515$ and 14.584 ± 1.298 , respectively) (Figure 2.13B), where AQP3 is the main contributor. Treatment with Auphen (10 μ M, 90 min at RT) reduced around 50% P_{gly} ($(0.021 \pm 0.003) \times 10^{-4}$), as found in other cell systems (Figure 2.13A) [46,47].

In vivo, when monocytes are recruited by injured tissues and differentiate in macrophages or dendritic cells, their phenotype and physiology changes. Likewise, *in vitro*, by attaining macrophage phenotype, resting THP-1 cells end up adhering to the plates. Thus, to evaluate P_f and P_{gly} in resting, non-activated and simultaneous in LPS-activated THP-1 cells, we directly assayed cell volumes changes of individual adherent cells loaded with calcein, by epifluorescence microscopy as previously described [25,48,49,50,51]. In a perfusion chamber, cells are exposed to a hyperosmotic mannitol buffer and the consequent water outflow induces cell shrinkage (Figure 2.13C). Water permeability P_f is evaluated by monitoring the time course of fluorescence output that reflects the transient volume change (V_{rel}). As shown in Figure 2.13C, similar rates of volume equilibration were observed when comparing different conditions. The calculated P_f values of LPS-activated cells ($(1.624 \pm 0.335) \times 10^{-3} \text{ cm s}^{-1}$) are statistically similar to the values for resting ($(1.188 \pm 0.435) \times 10^{-3} \text{ cm s}^{-1}$) and non-activated cells ($(0.954 \pm 0.206) \times 10^{-3} \text{ cm s}^{-1}$) (Figure 2.13H). To assess glycerol permeability P_{gly} , cells are challenged with a glycerol hyperosmotic solution and the time course of cell volume equilibration (V_{rel}) is monitored. After the first fast cell shrinkage due to water outflow, glycerol influx is followed by water with consequent cell reswelling (Figure 2.13D). Although LPS activation impaired AQP3 mRNA expression levels, similar rates of glycerol influx were detected in resting, non-activated and LPS-activated cells (Figure 2.13D). The calculated P_{gly} for resting cells ($(1.700 \pm 0.442) \times 10^{-6} \text{ cm s}^{-1}$) was similar to the measured in non-activated ($(1.454 \pm 0.350) \times 10^{-6} \text{ cm s}^{-1}$) and LPS-activated cells ($(1.242 \pm 0.412) \times 10^{-6} \text{ cm s}^{-1}$) (Figure 2.13I), indicating that LPS does not affect glycerol permeability. The obtained P_{gly} values are within the range of the measured in other mammalian cell line with high aquaglyceroporins endogenous

expression [51,52,53], possibly reflecting the main AQP3 contribution for glycerol transport in THP-1.

Although AQP3 mRNA expression is upregulated with LPS priming, differentiating monocyte-like cells into macrophages with PMA and activating them with LPS did not increase water or glycerol permeability. So, we checked AQP3 protein expression in these three conditions. Figure 2.13G depicts our results that show similar levels of AQP3 protein in monocyte-like cells, PMA-differentiated macrophages-like cells and LPS-activated macrophages like-cells.

Subsequently, cells were treated with Auphen (30 min at 37°C) before permeability assays. Water permeability was not statistically significant between cells conditions ($(1.196 \pm 0.040) \times 10^{-3} \text{ cm s}^{-1}$ for resting cells; $(1.124 \pm 0.650) \times 10^{-3} \text{ cm s}^{-1}$ for macrophages-like cells; and $(1.042 \pm 0.412) \times 10^{-3} \text{ cm s}^{-1}$ for LPS-activated cells; Figure 2.13E). However, glycerol permeability was substantially reduced in all cells ($(0.445 \pm 0.008) \times 10^{-6} \text{ cm s}^{-1}$ for resting cells; $(0.103 \pm 0.014) \times 10^{-6} \text{ cm s}^{-1}$ for non-activated cells; and $(0.185 \pm 0.015) \times 10^{-6} \text{ cm s}^{-1}$ for LPS-activated cells; Figure 2.13F), with a similar 50% reduction as observed by stopped-flow, and validating AQP3 as the main active glycerol channel in THP-1 cells.

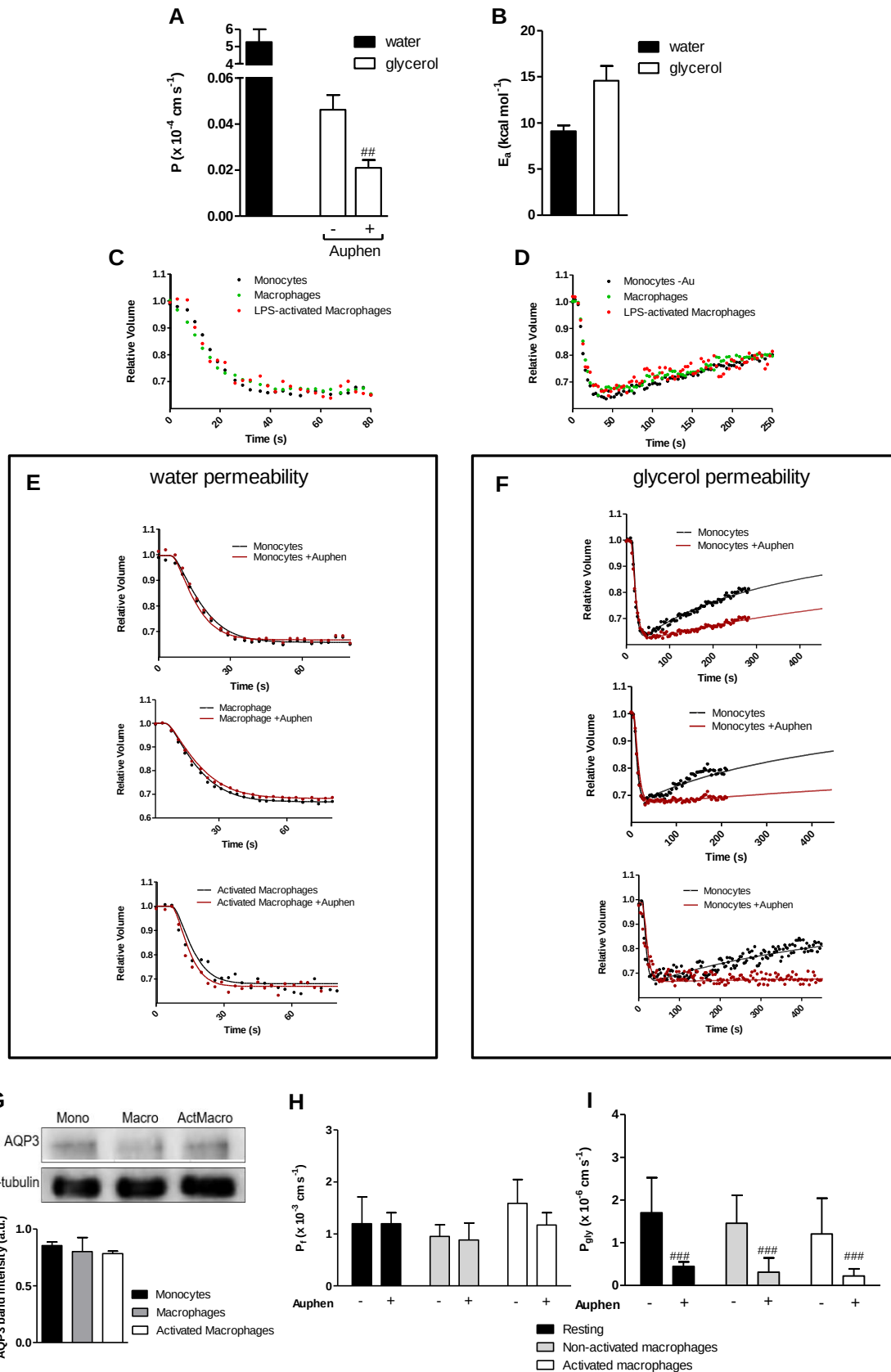


Figure 2.13. AQP3 is a functional glycerol channel in THP-1 and is inhibited by Auphen. (A) Water and glycerol permeabilities measured by stopped-flow. (B) Activation energy for water and glycerol transport. (C) Time course of volume change after an osmotic challenge

with mannitol and **(D)** glycerol detected in adherent cells by fluorescence microscopy. **(E)** Representative water and **(F)** glycerol permeability measurements in monocytes, macrophages and activated macrophages before and after Auphen treatment. **(G)** Representative blots and relative AQP3 protein expression in monocytes (Mono), macrophages (Macro) and activated macrophages (ActMacro) in relation to α -tubulin. **(H)** Water permeability (P_f). **(I)** Glycerol permeability (P_{gly}). Bars show mean $\pm$ SD from 10 cells analyzed on 3 coverslips in 2 cell platings. ##, $P<0.01$, ###, $P<0.001$; #, Auphen-treated vs non-treated cells.

AQP3 is involved in the effectiveness IL-6 release

The functional activation of the NLRP3 inflammasome requires a first signal (signal 1), or cell priming, that may precede or coincide with a secondary NLRP3-specific activating signal (signal 2) [54]. Cell priming, accomplished by NF- κ B-activating receptors such as TLRs, cytokine receptors and TNF receptor (cell death receptors), results in activation and nuclear translocation of NF- κ B, nuclear factor that upregulates NLRP3, whose endogenous levels are inadequate for inflammasome activation, pro-IL-1 β and Caspase-1, because their expression is not constitutive. Signal 1 is also responsible to the posttranslational regulation of inflammasome components including NLRP3 and ASC ubiquitination and phosphorylation [55]. Furthermore, NF- κ B can bind to the promoter region of IL-6 gene promoting its transcription, and consequently, as IL-6 maturation does not require cleavage by a caspase, IL-6 release has been used as a validation that effective priming occurred.

The triggers for NF- κ B-activating receptors might be a variety of stress or injury signals, such as PAMPSs or DAMPs. Here, to achieve cell priming, we exposed TLR4, a member of TLR family, to LPS eliciting a pro-inflammatory response. By triggering NF- κ B signaling pathway, IL-6 and pro-IL-1 β mRNA expression levels are augmented, while only IL-6 protein is release owing to the lack in caspase maturation till the signal 2 is sensed. Our results, showed an upregulation in IL-6 ($(26.4\pm 2.97) \times 10^{-5}$) and pro-IL-1 β (9.53 ± 0.551) mRNA expression levels in LPS-activated cells when incubating with LPS for 24h when comparing to non-activated cells ($(4.39\pm 2.27) \times 10^{-5}$ for IL-6; and 3.637 ± 0.218 for IL-1 β), validating our model (Figure 2.14A).

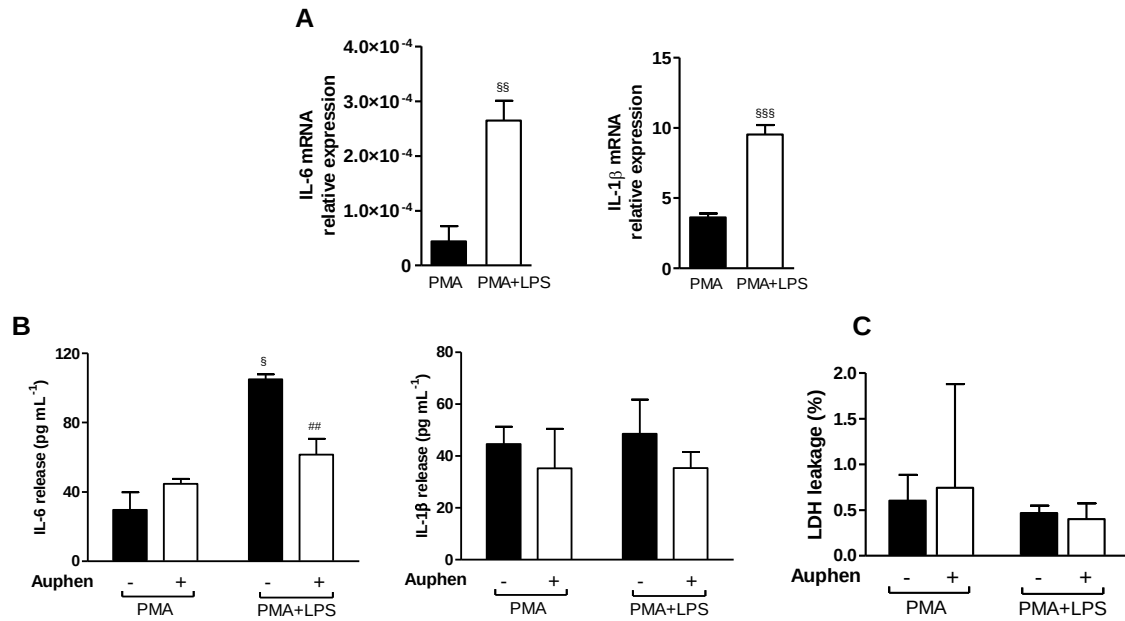


Figure 2.14. AQP3 involvement in macrophage priming. (A) mRNA expression levels of IL6 and IL1β in non-activated (PMA) and activated macrophages (PMA+LPS). (B) IL6 and IL1β protein release in non-activated (PMA) and activated macrophages (PMA+LPS) non-treated and treated with Auphen. (C) Percentage of cell death by pyroptosis. Bars show mean ± SD from 3 independent experiments. ##, P<0.01; #, Auphen vs without Auphen. §, P<0.05, §§, P<0.01, §§§, P<0.001; §, LPS vs PMA.

When looking at the protein secretion level, an increase in IL-6 protein release was measured after LPS incubation (105 ± 2.376 pg mL⁻¹) comparing to non-activated cells (29.733 ± 8.250 pg mL⁻¹) (Figure 2.14B). Interestingly, our results showed that Auphen, the AQP-specific inhibitor, when incubated along with LPS-priming, the AQP3-specific inhibitor, partially blocks IL-6 protein (61.533 ± 7.448 pg mL⁻¹; Figure 2.14B). On the other hand, no statistical differences were observed in IL-1β protein release after LPS-priming (44.547 ± 5.479) as we expected comparing to non-primed cells (48.483 ± 10.839 pg mL⁻¹) (Figure 2.14B). Figure 2.14C depicts a toxicity assay, enlightening the lack of cytotoxicity associated with the use of Auphen. Altogether, our data suggest that AQP3 is involved in IL-6 release during cell priming via TLR4. Furthermore, the exact mechanism for IL-6 release is poorly understood and here we show that AQP3 plays a role in this pathway. It was previously demonstrated that IL-6, at both gene and protein level, is induced by IL-1 in mouse primary cortical neurons, however, it is the cell depolarization that cause transport and release of IL-6 vesicles

[56]. Interestingly, AQP7, other aquaglyceroporin and the most representative isoform in pancreatic β -cells, has been shown to play an important role in the exocytosis pathway of insulin [57,58]. In this study, it was demonstrated that a raise in blood glycerol induces pancreatic β -cell swelling, activating volume-regulated anion channels (VRAC) and subsequent chloride efflux and cell membrane depolarization. The depolarization opens voltage-sensitive calcium channels and calcium enters, triggering a signalization cascade that results in insulin secretion [57].

AQP3 inhibition reduces IL-1 β release via NLRP3

The signal 2 for inflammasome activation, which leads to NLRP3-specific activation, assembling of the NLRP3 inflammasome complex and completes the activation of Caspase-1 culminating in the secretion of mature IL-1 β and IL-18 can be trigger by a variety of triggers. NLRP3-inflammasome can be signalized to ensembled by crystals requiring phagocytosis, ATP via cell surface purinergic P2X7 receptor, and pore-forming toxins such as nigericin [9]. Here, to attain effective inflammasome activation, we incubated cells with hyperosmotic solution of glycerol, nigericin and ATP in independent experiments causing pro-casp1 maturation and activation and consequent IL-1 β cleavage and release. A THP-1 caspase-1 knockdown strain was used parallely as a negative control for pyroptosis. To challenge AQP3 function, cells were incubated with Auphen simultaneously with the signal 2 and the functional inhibition was then evaluated. Our results showed that, challenging LPS-activated cells with a hyperosmotic solution of glycerol IL-1 β release increased (263.57 ± 46.22 pg mL⁻¹) comparing with non-challenged cells (37.917 ± 0.372 pg mL⁻¹), furthermore, this release was reduced by Auphen (113.67 ± 35.02 pg mL⁻¹; Figure 2.15A). Cell death evaluation results are in agreement with IL-1 β release data, where percentage of pyroptosis was higher in challenged cells (6.360 ± 0.839) compared to non-challenged cells (0.782 ± 0.374). In addition, cell death was partially reduced in Auphen-treated cells (3.736 ± 0.475) comparing with Auphen non-treated cells (0.315 ± 0.361 ; Figure 2.15B). IL-1 β release or cell death observed in THP-1 Casp-1 KO cells were neglectable (Figure 2.15A and B).

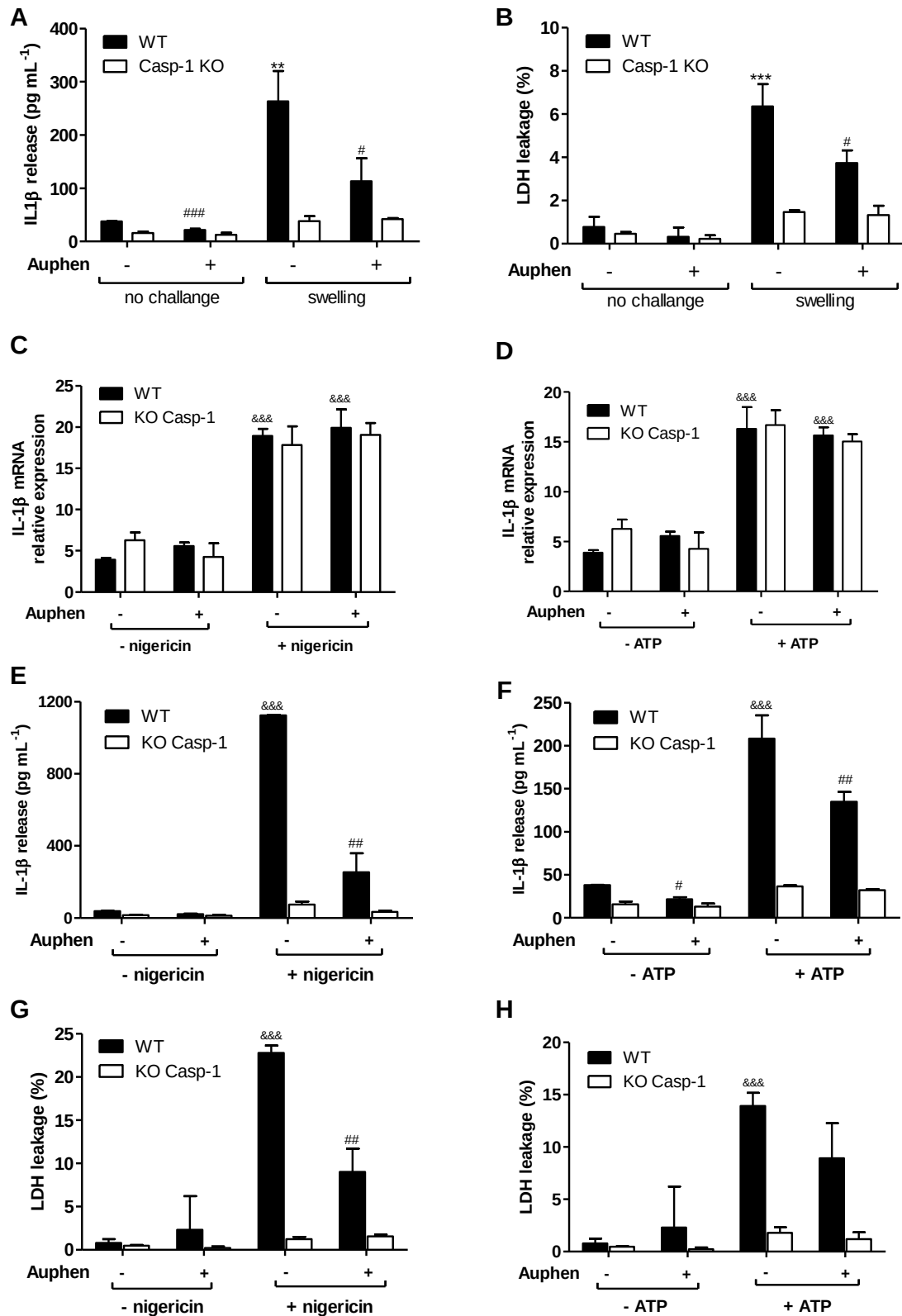


Figure 2.15. Contribution of AQP3 for inflammasome activation. (A) IL1 β release and (B) percentage of cell death by pyroptosis after imposing cell swelling in cells non-treated and treated with Auphen. (C and D) IL-1 β mRNA expression relative to HPRT-1; (E and F) IL1 β protein release, and (G and H) percentage of cells that suffered pyroptosis after challenging

cells with nigericin (**C**, **E** and **G**) and ATP (**D**, **F** and **H**). All experiments were done in wild-type (WT) and Caspase-1 knocked-out (Casp-1 KO) cells non-treated and treated with Auphen. Bars show mean $\pm$ SD from 3 independent experiments. **, $P < 0.01$, ***, $P < 0.001$; #, swelling vs no challenge. #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.001$; #, Auphen vs without Auphen. &&&, $P < 0.001$; &, treatment (nigericin or ATP) vs LPS.

When adding nigericin or ATP to LPS-activated cells, both IL-1 β mRNA (18.93 $\pm$ 0.682 and 16.33 $\pm$ 1.772, respectively) and protein release (1124.7 $\pm$ 2.082 and 208.27 $\pm$ 22.22) were increased in WT THP-1 cells (3.913 $\pm$ 0.182 for mRNA; and 37.917 $\pm$ 0.372 pg mL⁻¹ for protein release), attesting inflammasome activation and consequent IL-1 β maturation and release (Figure 2.15C). Interestingly, when cells were incubated with Auphen, the amount of released protein was impaired in both nigericin (253.27 $\pm$ 86.72 pg mL⁻¹) and ATP (134.87 $\pm$ 9.402 pg mL⁻¹) conditions (Figure 2.15E and F, respectively). Cell death evaluation results are in agreement with IL-1 β release data, where percentage of pyroptosis was higher in nigericin (22.808 $\pm$ 0.677) or ATP (13.930 $\pm$ 1.022)-challenged cells compared to non-challenged cells (0.782 $\pm$ 0.374). In addition, cell death was partially reduced in Auphen treated cells (9.006 $\pm$ 2.199 for nigericin; and 8.918 $\pm$ 2.738 for ATP) comparing with Auphen non-treated cells (2.315 $\pm$ 3.190; Figure 2.15G and H). As with the hyperosmotic solution challenge, IL-1 β release or cell death observed in THP-1 Casp-1 KO cells were neglectable (Figure 2.15C-H). Our results also point, a stronger activation of the inflammasome by nigericin, comparing with ATP or hyperosmotic solution of glycerol as a second signal. Overall, this results strongly support the involvement of AQP3 in IL-1 β release via NLRP3-inflammasome activation through a variety of stimuli, such as glycerol, nigericin and ATP.

Cytosolic K⁺ is not changed by cell swelling via AQP3

NLRP3-inflammasome activation by a K⁺ efflux has been reported [14], and a number of conditions that deplete cytosolic K⁺ revealed to activate NLRP3, such as inhibition of Na⁺/K⁺-ATPase with dissipation of membrane potential and variation of extracellular and intracellular osmolarities [59]. In order to investigate if cell swelling via AQP3 is an event dependent on K⁺ efflux for NLRP3 activation, we measured the intracellular K⁺ content after challenging cells with glycerol hyperosmotic and

hypoosmotic solutions. We activated the inflammasome with nigericin as a control of decreased cytosolic K^+ content (0.767 ± 0.170 mM). Our results showed no variation in internal K^+ after cells were challenged with hyperosmotic (1.188 ± 0.063 -fold change) and hypoosmotic solutions (0.917 ± 0.116 -fold change) comparing to non-challenged cells (1.104 ± 0.055 -fold change) (Figure 2.16). Therefore, cell swelling via AQP3 does not contribute to the cytosolic K^+ decrease described to activate NLRP3-inflammasome.

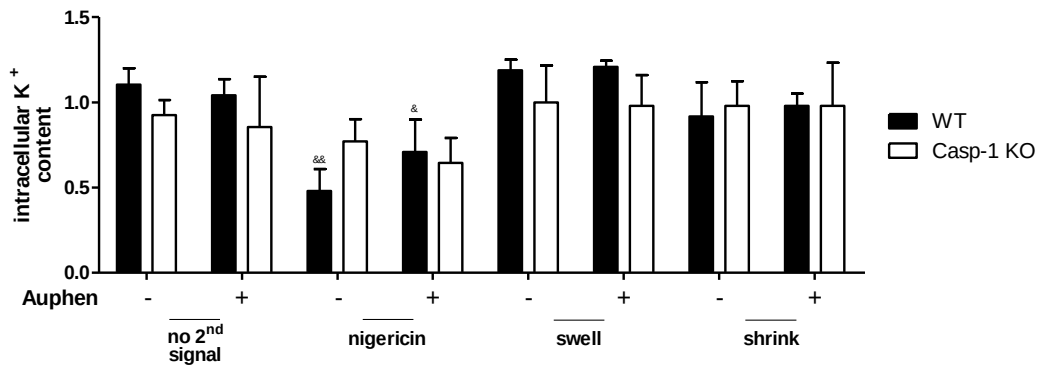


Figure 2.16. AQP3-dependent inflammasome activation does not affect intracellular K^+ . Intracellular K^+ content in response to nigericin, swelling and shrinking as 2nd signal for NLRP3-inflammasome activation in non-treated and Auphen-treated WT and Casp-1 KO cells and normalized to control cells. Data represent a mean of three independent experiments. &, $P < 0.05$, &&, $P < 0.01$; &, 2nd signal (nigericin, swell or shrink) vs no 2nd signal.

AQP3 inhibition affects in IL-1 β release

Signal 2 sensing leads to NLRP3-inflammasome assembly and Caspase-1 activation [54]. Once Caspase-1 is activated, proIL-1 β is processed in its mature form, IL-1 β , that is then released mainly by pyroptosis. To understand the exact mechanism in which AQP3 plays a role in inflammasome activation, we evaluated the levels of internal proIL-1 β and IL-1 β after giving cells, treated and nontreated with auphen, a second signal (nigericin and swelling). Similar levels of proIL-1 β were detected when adding nigericin (0.751 ± 0.130) or inducing swelling (0.644 ± 0.086), comparing to control cells (0.566 ± 0.082) (Figure 2.17). However, when we look at the protein levels of mature IL-1 β , knowing that IL-1 β release already occurred (as shown in Figure 2.15E), we observe that Auphen treated cells show high levels of IL-1 β intracellular accumulation when challenged with nigericin (0.593 ± 0.120) or swelling (0.623 ± 0.180),

when comparing with non-treated cells (0.104 ± 0.013 and 0.367 ± 0.087 , respectively). Thus, these data strongly suggest that AQP3 is involved in IL-1 β release.

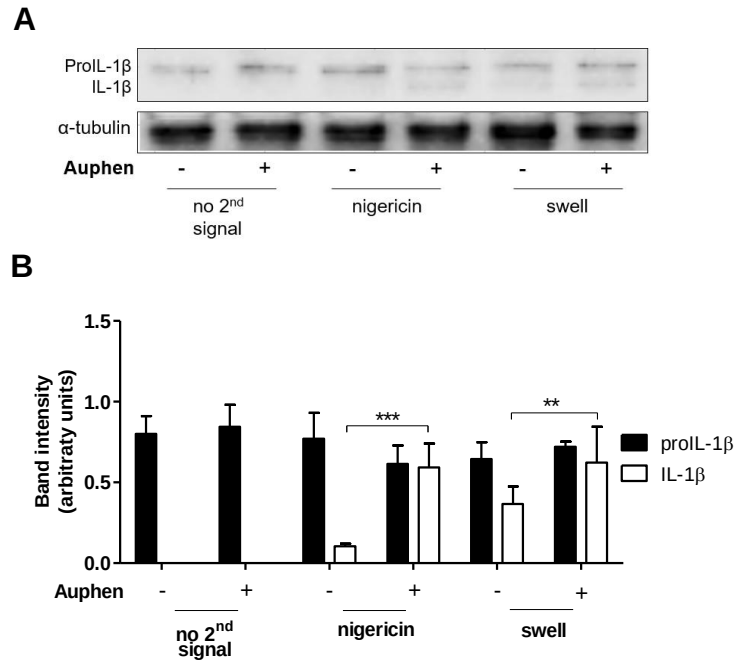


Figure 2.17. AQP3 is needed for IL-1 β release. (A) Representative blot and (B) relative proIL-1 β and IL-1 β protein expression normalized to α -tubulin, showing intracellular proIL-1 β and IL-1 β protein in response to nigericin and swelling in non-treated and Auphen-treated activated macrophages. Data represent a mean of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$; *, with Auphen vs without Auphen.

Discussion

Aquaporins are key players in cell swelling and volume regulation. Previous studies have suggested the involvement of aquaporins in inflammasome activation. Recently, evaluation of mRNA and protein expressions showed the presence of a few aquaporin isoforms in several immune cells, such as monocytes and macrophages [32,38,39,43,44,45]. Here, we report the mRNA expression of AQP9 as the most representative isoform in human primary monocytes and macrophages, confirming previous studies [43]. In addition, AQP3, AQP6, AQP8, AQP11 and AQP12 were also detected although in lower amounts. Interestingly, besides being the most expressed in macrophages, AQP9 is also upregulated in LPS-activated macrophages in parallel with inflammation biomarkers such as IL-6, IL-1 β and TNF α (Figure 2.12). The

upregulation of AQP9 by LPS priming was previously reported in macrophages and also in neutrophils at both mRNA and protein levels [43].

The present study was conceived to investigate the potential involvement of aquaporins in NLRP3-inflammasome priming and activation as a critical step in the development of inflammatory process. NLRP3-based inflammation has been associated with several diseases, including metabolic homeostasis dysregulation, such as type-2 diabetes, fatty liver disease and gout [1,2,3,4,5]. The dysregulation in AQP9 mRNA expression suggest that this isoform plays a part on the onset of inflammation although the mechanism is still obscure. Detecting aquaporin function in primary macrophages has shown to be challenging. The lack of AQP9 inhibitors, the high toxicity of the general inhibitor HgCl₂ and the limitations in generating knockdowns in these cells made us use a different model. With THP-1 cells we are able to obtain macrophages-like cells by differencing *in vitro* monocyte-like cells with PMA, and we can induce activation with LPS, making this cell line a useful model to study inflammation. In THP-1 cells we detected AQP1, AQP3, AQP5, AQP7, AQP8, AQP9 and AQP11 mRNA expression, being AQP3 the most expressed isoform and also upregulated by LPS-priming (Figure 2.12). However, the same upregulation was not observed at protein level, since resting, macrophages-like and activated macrophages like-cells showed similar levels of AQP3.

Rapid volume changes characterize inflammasome activation during an inflammatory response [13], so water and glycerol permeability of THP-1 resting, macrophages-like and LPS activated macrophages like-cells were investigated. Our results showed that THP-1 differentiation and priming/activation are associated with unaltered water and glycerol transport (Figure 2.13). Although these observations suggest that the upregulation of aquaglyceroporins mRNA expression is associated with the establishment of a pro-inflammatory phenotype, in this model, AQPs protein levels and consequent function were not altered. Thus, since AQP3 is the most predominant in THP-1, we further validated its activity as glycerol channel by two distinct methods, stopped-flow and fluorescent microscopy. Both methods revealed around 50% reduction of glycerol permeability when THP-1 cells were treated with Auphen, similar to the observed in other cell lines [46,49], validating AQP3 as a functional aquaporin in these cells.

Two signals are required for NLRP3-inflammasome activation, cell priming/activation (signal 1) and inflammasome activation (signal 2) [54]. In

macrophages, LPS-priming is accomplished by NF- κ B-activating receptors such as TLRs, resulting in the activation and nuclear translocation of NF- κ B, a nuclear factor that upregulates NLRP3, IL-6, pro-IL-1 β , pro-IL-18 and caspase-1. IL-6 maturation does not require cleavage by a caspase, contrary to IL-1 β and IL-18, so its release is used as a validation for effective priming. Here, we attested an upregulation in IL-6 mRNA and protein release and in pro-IL-1 β mRNA expression levels when priming cells with LPS validating our model (Figure 2.14A). Our results highlight the importance of AQP3 in cell priming, when we block AQP3 and IL-6 protein release is reduced comparing to cells non-treated with Auphen (Figure 2.14B), suggesting that AQP3 is involved in IL-6 release during cell priming via NF- κ B. Although the exact mechanism for IL-6 release is poorly understood, here we show that AQP3 plays a role in this pathway. Previous studies demonstrated that transport and release of IL-6 vesicles is induced by cell depolarization [56]. Interestingly, AQP7, other aquaglyceroporin, has been shown to play an important role in the exocytosis pathway of insulin in pancreatic β -cells by generating cell depolarization [57,58].

Inflammasome activation (signal 2) leads to the assembly of the NLRP3-inflammasome complex and completes the activation of Caspase-1, processing of proIL-1 β and IL-18 and ending in the secretion of mature IL-1 β and IL-18 mainly by pyroptosis, also triggered by active caspase-1 [9]. Here, we challenge LPS-primed cells with nigericin (positive control) and hyperosmotic solution of glycerol to induce cell swelling in order to clarify if AQP3 is involved in inflammasome activation. As previously described for THP-1, nigericin promoted IL-1 β release, meaning that NLRP3-inflammasome was activated, caspase-1 turned into its active form and processed proIL-1 β and promoted pyroptosis, also attested by high levels of LDH in medium. Our results showed for the first time that cell swelling via AQP3 can induce inflammasome activation by itself. In addition, the functional inhibition of AQP3 leads to a reduction in IL-1 β release (Figure 2.15). These results suggest that AQP3, although with lower specificity for NLRP3-inflammasome than nigericin, can induce NLRP3-inflammasome assembly and activation.

Previous experiments have already described an increase in IL-1 β mRNA and protein release when challenging LPS-activated cells with nigericin and ATP as signal 2, and here, we could verify the same results (Figure 2.15). Interestingly, although IL-1 β mRNA expression was not altered, the amount of released protein was impaired in both nigericin and ATP conditions when AQP3 was blocked (Figure 2.15) pointing an

involvement of AQP3 upstream to interleukin genes transcription. Our results also show a stronger activation of NLRP3-inflammasome by nigericin, comparing with ATP or hyperosmotic solution of glycerol as a second signal. Overall, this results strongly support the involvement of AQP3 in the mechanism of IL-1 β release via NLRP3-inflammasome through a variety of stimuli, such as glycerol, nigericin and ATP. Despite using different approaches, our findings are in agreement with prior published data. Other studies indirectly assessed aquaporin involvement. An evaluation of the link between cell volume changes and IL-1 β release by inhibiting water fluxes using HgCl₂ showed the need of cell swelling for inflammasome activation [13].

The exact mechanism by which AQP3 interferes with inflammasome priming and activation might not have been totally clarified, however, our data showed that the characteristic K⁺ efflux that is commonly observed prior to inflammasome activation is not a consequence of cell swelling (Figure 2.16), so it might happen as a downstream event to AQP3-driven water efflux as reported in previous studies [60].

Caspase-1 is activated with inflammasome activation and is then able to process proIL-1 β in its mature form, IL-1 β , that is then released mainly by pyroptosis [9]. Here, we investigated the amount of intracellular protein of proIL-1 β and IL-1 β after challenging LPS-activated macrophages like-cells with a second signal (nigericin and swell). Our results show similar levels of proIL-1 β , however, when we look at the mature IL-1 β protein levels, we observe that cells treated with Auphen show high levels of IL-1 β intracellular accumulation when challenge cells with nigericin or swelling, suggesting that AQP3 might be involved in IL-1 β release since cells whose AQP3 was inhibited showed trapped intracellular mature IL-1 β (Figure 2.17).

Conclusions

Aquaporins were previously associated with different physiological cellular processes in innate and adaptive immunity, such as motility, proliferation and activation. In addition, cell activation is related to the upregulation of the most representative isoform in the cell, suggesting a link between AQP dysregulation and disease [43]. Our results support the upregulation of AQP9 and AQP3 upon LPS-priming in HPBM and THP-1 cells, respectively. Despite the AQP3 increased mRNA levels in activated cells, total cell aquaporin function is not altered in THP-1, however,

it was shown to be a good model to study the setting of inflammasome activation because we could modulate AQP3 function using AQP3-specific inhibitor Auphen. Our results show that AQP3 is involved in TLR4 activation mechanism impairing IL-6 release when blocked. In addition, the reduction of IL-1 β release when inhibiting AQP3 reveal its involvement in NLRP3-inflamassome activation. Despite the need of further investigation to clarify this hypothesis, our results suggest that AQP9, being the most expressed isoform in primary monocytes, is a candidate player in the setting of NLRP3-inflammasome activation with implications in various disease. Inflammation has been linked with the development of several pathologies that til date seemed to be independent. A strong link between inflammation and metabolism is becoming increasingly evident and the activation of the NLRP3 inflammasome has been implicated in a variety of metabolic diseases including obesity, atherosclerosis and type-2 diabetes. Altogether, this new information underlying the mechanisms of AQPs in the regulation of the inflammatory response opens up new scenarios to tailor the modulation of specific AQPs as target for new pharmacological therapies.

Material and Methods

Reagents

Key reagents and their sources were as follows: Escherichia coli LPS serotype 055:B5, nigericin sodium salt, MCC950 (CP-456773), ATP, bovine serum albumin. The composition of the physiological buffer used in all experiments to stimulate cells was as follows: 147 mM NaCl, 10 mM HEPES, 13 mM D-glucose, 2 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂; pH 7.4.

Human peripheral blood monocytes (HPBM) isolation and treatment

HPBM were isolated from healthy donors' whole peripheral blood (n=3) by negative selection using the EasySep™ Cell Separation human monocyte enrichment cocktail (StemCell Technologies), according to the manufacturers' instructions. The Institutional Review Board of the Hospital Clínico Universitario Virgen de la Arrixaca approved the use of these blood samples. Informed consent was obtained from all individuals enrolled in the study following the principles set out in the WMA Declaration of Helsinki. Monocytes were cultured in complete medium (RPMI 1640 medium (Biowest) supplemented with 1% L-Glutamine, 10% FBS (Hyclone), no antibiotics. For macrophage differentiation, cells were plated at a density of 0.5M monocytes/well P24 and left to adhere and were then treated with LPS 100ng/mL for 2h.

Control cells were left untreated. These cells were used to screen all aquaporin isoforms expression and its dynamics upon LPS-activation.

THP1 cells culture and treatments

THP-1 wild-type (WT) and knockout for Caspase-1 (KO Casp-1) were cultured in complete medium (RPMI 1640 medium (Biowest) supplemented with 1% L-Glutamine, 10% FBS (Hyclone), no antibiotics at 37°C with 5% CO₂. For the experiments, cells were plated at a density of 2M cells/well P6. To differentiate them we used PMA 0.5uM for 30min and for priming LPS 100ng/mL for 24h. In order to understand the mechanisms entangled with inflammasome activation, nigericin 10 µM for 30 min or AQP 3mM for 30 min were added to the media as a second signal. To inhibit AQPs function, Auphen mM was added to the media simultaneously with the specific signal under investigation (to study AQP influence in the first signal, cell priming, Auphen was added with LPS for 24 hours; to study AQP influence in the second signal, inflammasome activation, Auphen was added with nigericin or ATP for 30min). These cultures were used as an alternative for primary cultures due to the difficulty to ablate AQP function in those cells (by transfection or functional inhibitors). THP-1 WT cells were used to screen AQPs isoforms expressed in resting monocyte-like cells, non-activated macrophages-like cells and LPS-activated macrophages-like cells. Cells were then used in permeability assays by stopped flow light scattering and/or fluorescent microscopy, and to investigate about the involvement of AQPs in the transduction of the first and second signal for inflammasome activation. THP-1 KO Casp-1 were used in parallel with THP-1 WT, as a negative control, in experiments involving second signal for inflammasome activation.

RNA extraction and quantitative PCR

HPBM were washed twice with PBS before total RNA purification using the MicroRNeasy kit (Qiagen) and treated with TurboTMDNase (Invitrogen) for 30min to remove any trace of genomic DNA, according to the manufacturer's recommendations and quantified on a NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription was performed using an iScriptTM cDNA Synthesis Kit (BioRad). qPCR was performed using an iQTM 5 Optical System (BioRad) with a SYBR Green mix (Takara) and specific pre-designed gene expression primers (Qiagen).

THP-1 cells were washed twice with PBS before total RNA purification using the RNeasy mini kit (Qiagen) and treated with RNase-free DNase I (Invitrogen) for 30min to remove any trace of genomic DNA, according to the manufacturer's recommendations. Retrotranscription was performed as described for HPBM. Real-time PCR reactions were carried out using a CFX96 Real-Time System C1000 (BioRad), the TaqMan Universal PCR Master Mix (Applied Biosystems) and the following specific TaqMan pre-designed gene expression primers: AQP0

(Hs0085175_m1), AQP1 (Hs01028916_m1), AQP2 (Hs00166640_m1), AQP3 (Hs01105469_g1), AQP4 (Hs00242342_m1), AQP5 (Hs00387048_m1), AQP6 (Hs00155808_m1), AQP7 (Hs00357359_m1), AQP8 (Hs01086280_g1), AQP9 (Hs00175573_m1), AQP10 (Hs00369738_m1), AQP11 (Hs005426181_m1), AQP12 (Hs01651303_m1), HPRT-1 (Hs02800695_m1), IL6 (Hs00174131_m1) and IL1 β (Hs01555410_m1) (Applied Biosystems). All results were normalized to the level the reference gene (HPRT1) and relative quantification was calculated using a variation of the Livak method [61], described by Fleige & Pfaffl [62]. All samples were run in triplicate and the average values were calculated.

Western Blotting

Proteins were separated by SDS-PAGE before semi-dry transfer as previously described [51]. After blocking, the following specific primary antibodies were used: anti-AQP1 and AQP3 antibody (1:100 and 1:200, respectively; Santa Cruz Biotechnology), anti-IL-1 β and anti- α -tubulin (1:1000; Sigma–Aldrich) antibody. Secondary anti-rabbit and anti-mouse (1:5000; Jackson Immuno Research) and anti-goat (1:7500; Jackson Immuno Research) HRP-linked antibodies were used for sensitive ECL-mediated (Amersham Biosciences) detection. Band intensity was measured using the ImageJ software (<https://imagej.nih.gov>).

Permeability assays in cell suspensions

THP-1 cells were centrifuged at 150 $\times$ g to obtain a cellular pellet. The cells were resuspended in isotonic medium and left for 10 min to reach the equilibrium in this medium. The cellular preparations were homogeneous and THP-1 cells were spherical in shape when in suspension, as observed under light microscopy. The diameter of cells was measured for all the preparations with ImageJ software with pictures obtained by light microscopy.

Permeability assays in suspension cells were performed by stopped-flow light scattering following the protocol described by Martins *et al.* [46]. Briefly, experiments were performed on a HI-TECH Scientific PQ/SF-53 stopped-flow apparatus, which has a 2 ms dead time and is temperature controlled (24°C), interfaced with an IBM PC/AT compatible 80386 microcomputer. This procedure was performed to measure water and glycerol permeability and the activation energy (E_a) for each transport, and to validate AQP3 as the representative glycerol channel in THP-1 using Auphen as inhibitor. For osmotic water permeability (P_f) measurements, a hyperosmotic shock solution containing a non-permeable solute was used (mannitol 660 mM in PBS 7.4) producing an inwardly directed gradient of solute. To measure glycerol permeability (P_{gly}), a hyperosmotic shock solution containing glycerol (glycerol 660 mM in PBS 7.4) creating an inwardly directed glycerol gradient. Four to eight runs were stored and analyzed in each experimental condition. After the first fast cell shrinkage due to water

outflow, glycerol influx in response to its chemical gradient was followed by water influx with subsequent cell re-swelling. In all the permeability assays the magnitude of the osmotic shocks (given by the ratio of the initial to final medium osmolarity after the applied osmotic challenges) is similar (tonicity of 1.6). Baselines were acquired using the respective incubation buffers as isotonic shock solutions. In each run 0.1 mL cellular suspension was mixed with an equal amount of hyperosmotic solution to reach inwardly directed gradients of solute.

The kinetics of cell shrinking and re-swelling were measured from the time course of 90° scattered light intensity at 530 nm until a stable light scatter signal was attained. Water (P_f) was calculated as $P_f = k (V_0/A)(1/V_w(\text{osmout})_\infty)$, where V_w is the molar volume of water, V_0/A is the initial cell volume to area ratio and $(\text{osmout})_\infty$ is the final medium osmolarity after the applied osmotic gradient and k is the single exponential time constant fitted to the light scattering signal of erythrocyte shrinkage; and glycerol permeability (P_{gly}) was achieved by $P_{gly} = k(V_0/A)$, where k is the single exponential time constant (s^{-1}) fitted to the light scattering signal of glycerol influx and V_0/A is the initial cell volume to area ratio. P_f and P_{gly} were measured in monocyte-like cells. In addition, cells were incubated with Auphen, an APQ3 specific inhibitor, Auphen (10 μM) was added to the cellular suspension 30 min before permeability measurements, according to previously published data [46]. The E_a of water and glycerol transport was calculated from the slope of the Arrhenius plot ($\ln P_f$ or $\ln P_{gly}$ as a function of $1/T$) multiplied by the gas constant R .

Permeability assays in adherent cells

Water (P_f) and glycerol (P_{gly}) permeability were measured in individual adherent cells on a coverslip, as previously described [25,50]. To perform this experiment in resting cells, coverslips were coated with poly-L-lysine for cell adherence. Briefly, cells were loaded with 5 mM calcein acetoxymethyl ester (calcein-AM; Sigma-Aldrich) for 30 min at 37°C in 5% CO_2 . The coverslips with the adhered cells were mounted in a closed perfusion chamber (Warner Instruments, Hamden, USA) on the stage of a Zeiss Axiovert 200 inverted microscope. Fluorescence was excited at wavelength 495/10nm and the emission fluorescence collected with a 535/25 nm bandpass filter coupled with a 515nm dichroic beam splitter. Images were captured using a $\times 40$ epifluorescence oil immersion objective and a digital camera (CoolSNAP EZ, Photometrics, USA) and recorded by the Metafluor Software (Molecular Devices, USA).

Cells were perfused with 300 mM HEPES buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-Glucose, 5 mM HEPES, pH 7.4, 300 mOsm) for 60 s, after which 300 mM mannitol (for water permeability) or 300 mM glycerol (for glycerol permeability) was added to the buffer to achieve an external osmolarity of 600 mOsm. Cell volume (V) was measured at selected time points from 2D images obtained during the permeability assays to evaluate the initial volume (prior to the osmotic challenge V_0) and the

final equilibrium volume [50]. P_f and P_{gly} coefficients were evaluated from the measured time dependent volume changes, $V_{rel} = V/V_o$, obtained by adding mannitol (P_f) or glycerol (P_{gly}) to the external media, using the model equations described by Madeira *et al.* [48] and the Berkeley Madonna software (<http://www.berkeleymadonna.com>).

Lactate dehydrogenase (LDH)

The presence of LDH in cell-free supernatants was measured using the cytotoxicity detection kit (Roche, Barcelona, Spain) following the manufacturer's instructions to evaluate treatment-dependent cell death. Cell death was defined as the percentage of released LDH compared with maximal LDH levels in cell lysates obtained using Lysis buffer on cells plated only with PMA (positive control). The percentage of the LDH release was calculated using ET buffer as negative control.

ELISA assays

Cell-free supernatants were collected and clarified by centrifugation. IL-6 and IL-1 β were quantified by ELISA following the kit supplier's protocol instructions (R&D Systems) and absorbances were measured in a Cobas 6000.

Internal K⁺ measurement

After stimulation with nigericin, hypertonic solution of glycerol (swelling) or hypertonic solution mannitol (shrinking) for 30 min, cells were washed briefly with molecular biology grade water (Sigma) to wash ions from the stimulating buffer. Cells were then scraped in 200 μ L molecular biology grade water and lysed by three freeze-thaw cycles. Lysates were centrifuged at 13200 rpm for 10 min at 4°C and supernatants were used to measure intracellular K⁺ content by direct potentiometry in a Cobas 6000 (Roche) according to the kit supplier's protocol (Roche).

Statistical analysis

All the experiments were performed in biological and technical triplicates. Results were expressed as mean $\pm$ SD of n individual experiments. Statistical analysis between groups was performed by two-way ANOVA and followed by a Student t-test. P values <0.05 were considered statistically significant. Statistical analyses were performed using the Graph Prism software (GraphPad Software).

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Chapter III

Genetic Polymorphisms in Cardiovascular Disease

3. Aquaporin and oxidative stress-associated genes polymorphisms may modulate cardiovascular risk in women

This subchapter is adapted from the following manuscript:

Inês Vieira da Silva, Ana Carolina Santos, Andreia Matos, Alda Pereira da Silva, Ana Portelinha, Maria José Areias, Graça Soveral, Irene Rebelo and Manuel Bicho. Aquaporin and oxidative stress-associated genes polymorphisms may modulate cardiovascular risk. (*in preparation*)

Abstract

Preeclampsia (PE) is a major public health issue, increasing the risk of hypertension (HT). Aquaporins are a family of water channels, comprising members that also transport hydrogen peroxide and/or glycerol, key molecules for redox signaling and metabolic homeostasis. Here, we study the association of aquaporin-3 (AQP3), aquaporin-7 (AQP7) and other oxidative stress-related genetic polymorphisms (NOS3, MPO, CYBA and CAT) with the development of PE and future HT in a cohort of 150 women normotensive (NT) or with PE. In addition, as a prospective study, the risk of developing HT in the future was assessed by following those women for 2-16 years after pregnancy. Anthropometric, hemodynamic parameters and oxidative stress-related biomarkers were determined. Our results show, for the first time, that AQP3 is a potential biomarker for the development of HT. Although AQP7 did not correlate with HT during or after pregnancy, it correlates with lipid profile status. The NOS3 polymorphism seems to be a good biomarker for PE, since its correlation with cardiovascular disease was only observed during pregnancy and not after it. CYBA polymorphism only predicts the development of HT after pregnancy implying two different mechanisms in the development of PE and HT after pregnancy. On one hand, NOS3 was associated with ischemia, while CYBA was more related to secondary HT and metabolic syndrome, through ROS modulation.

Keywords: Aquaporins, polymorphisms, oxidative stress, obesity, preeclampsia, hypertension, metabolic syndrome

Introduction

Preeclampsia, a pregnancy complication characterized by high blood pressure and proteinuria, which usually begins abruptly after 20 weeks of gestation, affects 7–10% of pregnancies worldwide [1]. The consequences of preeclampsia are not limited to pregnancy and may induce maternal permanent vascular, metabolic damage and future cardiovascular disease such as hypertension [2,3]. Preeclampsia and hypertension have been associated with inflammatory and oxidative stress, therefore, key proteins in redox signaling may be associated with inflammatory process and blood pressure biomarkers, which in turn may lead to the development of hypertension before and after pregnancy [4,5].

Aquaporins (AQPs) are a family of highly conserved transmembrane protein channels that facilitate the transport of water and small non-charged molecules (such as glycerol) across cell membranes [6,7]. In humans, 13 isoforms (AQP0-12) have been described with high amino acid sequence homology and tissue- and subcellular-specific localization, suggesting a link between the site of expression and physiological relevance [8]. AQPs are grouped based on their primary sequences and permeation selectivity. Orthodox AQPs (AQP0, 1, 2, 4, 5, 6, 8) are considered water channels, whereas aquaglyceroporins (AQP3, 7, 9, 10) also facilitate the movement of glycerol and other small non-charged solutes; and unorthodox AQPs (AQP11, 12) that are found mainly intracellularly, with lower sequence homology and permeability unclear [9]. In addition, a few members have been recently shown to facilitate H₂O₂ permeation across cell membranes were named peroxiporins [10,11].

Consistent with their wide tissue distribution, AQPs are associated with several physiological and pathophysiological processes [12]. In 2001, Damiano et al. detected AQP3 and AQP9 in the apical membrane of human syncytiotrophoblast [13]. Uptake experiments showed that these AQPs may mediate transcellular water, urea, and glycerol transport in the human placenta [14]. Lately, AQP3 expression has been reported to be impaired in preeclamptic placentas [15].

Apart from their metabolic commitment, where AQP7 and AQP9 work in synchrony in adipose tissue and liver, respectively, as glycerol channels, to maintain the whole body energy balance, AQP3 has been described as a H₂O₂ channel making it a key contributor to cell redox homeostasis and implicated in oxidative stress, a feature of Metabolic Syndrome [16,17,18]. Oxidative stress is characterized by an excessive production of reactive oxygen species (ROS) outstripping antioxidant defense mechanisms [19]. In humans, preeclampsia is also considered a state of oxidative stress that can contribute to the development of hypertension and other hypertension-induced organ damage such as atherosclerosis [20,21].

During pregnancy maternal blood pressure decreases to facilitate the blood flow towards the placenta where new vessels are being formed. The impaired blood pressure occurs as a result of a desensitization of angiotensin II receptors. The renin-angiotensin system is crucial in the maintenance of blood pressure and it is also involved vasculature formation and their fill with blood. Later in pregnancy, during fetus organ formation, maternal Angiotensin II receptors recover sensitivity in order to increase blood pressure and blood perfusion in maternal and fetal organs. Preeclampsia, and in

more severe cases eclampsia, is a result of improper maternal sensitization to vasoconstrictor agents. Hypertension in pregnancy commonly is associated with an improper placentation, ischemia/reperfusion and ROS production that will flow in the maternal bloodstream resulting in a hyperreactivity of the endothelial cells of the vessels leading to hypertension [2,3].

In hypertension, the mechanisms responsible for the increase of ROS species, O_2^- , H_2O_2 , and $\cdot OH$ are not yet fully understood, even though a mechanism, where an increase in the ROS production and/or a decrease in the disposal of antioxidant, have been proposed. Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), endothelial nitric oxide synthase (NOS3 or eNOS) and myeloperoxidases (MPO) are key enzymes that account for the majority of the ROS/RNS generation [22,23]. The role of NADPH oxidase as an important generator of ROS and the implication of eNOS during arginine and tetrahydrobiopterin deficiency were largely recognized in hypertensive states [22]. In addition, NOS3 is also responsible for NO synthesis with implications in endothelium health and vasodilatation. The main reactive species produced by MPO under physiological conditions are hypochlorous acid (HOCl) and species with nitrating activity in the presence of NO_2^- and H_2O_2 . Excessive generation of MPO-derived oxidants has been linked to tissue damage in many diseases, especially in those characterized by acute or chronic inflammation [24]. On the other hand, primary antioxidant enzymes in tissues, such as catalase (CAT), constitute an antioxidant defense system by detoxifying directly oxidizing species, contributing to cellular protection and maintaining the suitable intracellular redox state [25]. Thus, the activities of superoxide dismutase, catalase and glutathione peroxidase are reported to be significantly lower in hypertensive patients [23].

Cardiovascular disease and related abnormalities, including preeclamptic pregnancy and post gestational hypertension, are becoming epidemic thus representing major health care issues. Although these abnormalities have a clear genetic component, the involved genes are mostly to be identified. This prompted us to investigate the contribution of functional polymorphisms of AQP3 (rs2231231), AQP7 (rs2989924), NOS (intron-4), CYBA (rs4673; a subunit of NADPH oxidase), CAT (rs1001179) and MPO (rs2333227), individually or in combination, with the development of preeclampsia in Caucasian women that developed gestational hypertension compared to healthy women; and with the development of hypertension 2 to 16 years after pregnancy compared to normotensive women.

Results

In this cohort, we were able to evaluate polymorphisms-associated risk for several genes' key players in oxidative stress (AQP3, AQP7, NOS3, CYBA, MPO and CAT). The studies were first developed in pregnant Caucasian women, normotensive (NBPP) or with preeclamptic (PE) pregnancies. Then, based on a prospective study (2-16 years later), the same women were evaluated for the development of hypertension and classified as normotensive (NBPPP) or hypertensive (HT). The influence of genetic polymorphisms on the susceptibility for cardiovascular disease was then evaluated.

Table 3.1. Comparison of the distribution of genotype frequencies in women with normal blood pressure in pregnancy (NBPP) versus preeclamptic women (PE).

	Genotype	NBPP n=25 n(%)	PE n=56 n(%)	p value
AQP3	AA	2 (8.0)	9 (16.1)	0.392
	AC	9 (36.0)	24 (42.9)	
	CC	14 (56.0)	23 (41.0)	
AQP7	CC	9 (32.1)	15 (25.4)	0.790
	CT	12 (42.9)	29 (49.2)	
	TT	7 (25.0)	15 (25.4)	
NOS3	BB	35 (64.8)	68 (75.6)	0.067
	BA	13 (24.1)	20 (22.2)	
	AA	6 (11.1)	2 (2.2)	
CYBA	CC	4 (66.7)	12 (46.2)	0.505
	CT	2 (33.3)	10 (38.4)	
	TT	0 (0.0)	4 (15.4)	
MPO	GG	18 (40.0)	41 (53.2)	0.367
	GA	23 (51.1)	31 (40.3)	
	AA	4 (8.9)	5 (6.5)	
CAT	CC	37 (92.5)	54 (78.3)	0.125
	CT	3 (7.5)	12 (17.4)	
	TT	0 (0.0)	3 (4.3)	

Chi-square test. AQP3, Aquaporin-3; AQP7, Aquaporin-7; NOS3, Endothelial Nitric Oxide Synthase; CYBA, Cytochrome B-245 Alpha Chain; MPO, Myeloperoxidase; CAT, Catalase

NOS3 polymorphism is associated with the risk of developing preeclampsia

AQP3, AQP7, NOS3, CYBA, MPO and CAT polymorphisms were tested as biomarkers of susceptibility for the development of preeclampsia. Table 3.1 shows the genotypes distribution in NBPP and PE pregnant women ($N = 150$).

Table 3.2. Comparison of the distribution of genotype frequencies (dominant and recessive models) in women with normal blood pressure in pregnancy (NBPP) versus preeclamptic women (PE).

		Genotype	NBPP n=25 n(%)	PE n=56 n(%)	<i>p</i> value	OR [CI 95%]
AQP3	Dominant model of A	AA+AC	11 (44.0)	33 (58.9)	0.213	n.a.
		CC	14 (56.0)	23 (41.1)		n.a.
	Recessive model of A	AA	2 (8)	9 (16.1)	0.327	n.a.
		AC+CC	23 (92)	47 (83.9)		n.a.
AQP7	Dominant model of C	CC+CT	21 (75.0)	44 (74.6)	0.966	n.a.
		TT	7 (25.0)	15 (25.4)		n.a.
	Recessive model of C	CC	9 (32.1)	15 (25.4)	0.512	n.a.
		CT+TT	19 (67.9)	44 (74.6)		n.a.
NOS3	Dominant model of B	BB+BA	48 (88.9)	88 (97.8)	0.041	5.50 [1.07- 28.31]
		AA	6 (11.1)	2 (2.2)		1
	Recessive model of B	BB	35 (64.8)	68 (75.6)	0.167	n.a.
		BA+AA	19 (35.2)	22 (24.4)		n.a.
CYBA	Dominant model of C	CC+CT	6 (100.0)	22 (84.6)	0.304	n.a.
		TT	0 (0.0)	4 (15.4)		n.a.
	Recessive model of C	CC	4 (66.7)	12 (46.2)	0.365	n.a.
		CT+TT	2 (33.3)	14 (53.8)		n.a.
MPO	Dominant model of G	GG+GA	41 (91.1)	72 (93.5)	0.625	n.a.
		AA	4 (8.9)	5 (6.5)		n.a.
	Recessive model of G	GG	18 (40.0)	41 (53.2)	0.158	n.a.
		GA+AA	27 (60.0)	36 (46.8)		n.a.
CAT	Dominant model of C	CC+CT	40 (100.0)	66 (95.7)	0.181	n.a.
		TT	0 (0.0)	3 (4.3)		n.a.
	Recessive model of C	CC	37 (92.5)	54 (78.3)	0.054	n.a.
		CT+TT	3 (7.5)	15 (21.7)		n.a.

Chi-square test. AQP3, Aquaporin-3; AQP7, Aquaporin-7; NOS3, Endothelial Nitric Oxide Synthase; CYBA, Cytochrome B-245 Alpha Chain; MPO, Myeloperoxidase; CAT, Catalase; n.a., not applicable.

In our population of 150 pregnant women and concerning the evaluated genotypes distribution, no statistical differences were found between the genotypes distribution, of each of the six polymorphisms, between 60 normotensive (NBPP) and 90 PE women (Table 3.1).

Table 3.3. Comparison of the distribution of genotype frequencies in postpregnant women with normal blood pressure (NBPPP) versus hypertensive (HT) women

	Genotype	NBPPP n=47 n(%)	HT n=30 n(%)	p value
AQP3	AA	3 (6.4)	8 (26.7)	0.011
	AC	18 (38.3)	14 (46.6)	
	CC	26 (55.3)	8 (26.7)	
AQP7	CC	n=53 16 (30.2)	n=30 7 (23.3)	0.437
	CT	26 (49.0)	13 (43.4)	
	TT	11 (20.8)	10 (33.3)	
NOS3	BB	n=92 62 (67.4)	n=48 38 (79.2)	0.242
	BA	23 (25.0)	9 (18.7)	
	AA	7 (7.6)	1 (2.1)	
CYBA	CC	n=19 13 (68.4)	n=12 3 (25.0)	0.010
	CT	6 (31.6)	5 (41.7)	
	TT	0 (0.0)	4 (33.3)	
MPO	GG	n=74 35 (47.3)	n=45 21 (46.7)	0.912
	GA	34 (45.9)	20 (44.4)	
	AA	5 (6.8)	4 (8.9)	
CAT	CC	n=68 57 (83.8)	n=37 30 (81.1)	0.285
	CT	8 (11.8)	7 (18.9)	
	TT	3 (4.4)	0 (0.0)	

Chi-square test. AQP3, Aquaporin-3; AQP7, Aquaporin-7; NOS3, Endothelial Nitric Oxide Synthase; CYBA, Cytochrome B-245 Alpha Chain; MPO, Myeloperoxidase; CAT, Catalase.

Interestingly, when looking at NOS3 dominant model of B, we found that the BB+BA genotype was more frequent in PE (88.9%) than in NBPP women (11.1%) ($p=0.024$) (Table 3.2). Women carrying B allele present a risk of 5.50-fold ($p=0.041$, OR=5.50, 95% CI [1.07-28.31]) of developing PE during pregnancy, compared to

women with homozygotic AA (Table 3.2). No statistical differences were found between PE and NBPP frequencies were found for the other genetic polymorphisms studied.

AQP3 and CYBA genetic polymorphisms are associated with the risk of developing hypertension in women after pregnancy

AQP3, AQP7, NOS3, CYBA, MPO and CAT genetic polymorphisms were then tested as biomarkers of susceptibility to develop essential hypertension. Table 3.3 shows the genotypes distribution in a population of NBPPP and HT post-pregnancy women ($N = 146$).

In our population of 146 women, 98 normotensive women (NBPPP) and 48 HT, and regarding the evaluated genotypes distribution, we found an association between AQP3 ($p=0.011$) and CYBA ($p=0.010$) genotypes and the development of essential hypertension (Table 3.3).

In addition, our data showed that based on the AQP3 dominant model of A, AA+AC genotypes were more frequent (73.3%) in HT compared to CC (26.7%) ($p=0.014$) (Table 3.4). Moreover, in the dominant and recessive models of A, the allele A was considered the allele of risk, associated with a 3.405-fold higher risk ($p=0.016$, OR=3.405, 95% CI [1.26-9.19]) and 5.333-fold higher risk ($p=0.021$, OR=5.333, 95% CI [1.29-22.13]), respectively, to develop HT after pregnancy than the CC genotype in the dominant model and AC+CC genotype in the recessive model, respectively (Table 3.4).

Based on a dominant model of C, TT genotype of CYBA polymorphism was also correlated with the development of HT, only found in HT (100.0%) women in relation to CC+CT (0.0%) ($p=0.007$) after pregnancy (Table 3.4). On the other hand, looking at the recessive model of C, CT+TT genotype was associated with the development of HT. Furthermore, T was found to be the allele of risk associated with 6.500-fold risk ($p=0.024$, OR=6.500, 95% CI [1.28-33.03]) to develop HT after pregnancy, compared to CC genotype (Table 3.4).

Table 3.4. Comparison of the distribution of genotype frequencies (dominant and recessive models) in postpregnant women with normal blood pressure (NBPPP) versus hypertensive (HT) women

		Genotype	NBPPP n=47 n(%)	HT n=30 n(%)	p value	OR [CI 95%]
AQP3	Dominant model of A	AA+AC	21 (44.7)	22 (73.3)	0.016	3.405 [1.26-9.19]
		CC	26 (55.3)	8 (26.7)		1
	Recessive model of A	AA	3 (6.4)	8 (26.7)	0.021	5.333 [1.29-22.13]
		AC+CC	44 (93.6)	22 (73.3)		1
AQP7	Dominant model of C	CC+CT	42 (79.2)	20 (66.7)	0.205	n.a.
		TT	11 (20.8)	10 (33.3)		n.a.
	Recessive model of C	CC	16 (30.2)	7 (23.3)	0.503	n.a.
		CT+TT	37 (69.8)	23 (76.7)		n.a.
NOS3	Dominant model of B	BB+BA	85 (92.4)	47 (97.9)	0.181	n.a.
		AA	7 (7.6)	1 (2.1)		n.a.
	Recessive model of B	BB	62 (67.4)	38 (79.2)	0.143	n.a.
		BA+AA	30 (32.6)	10 (20.8)		n.a.
CYBA	Dominant model of C	CC+CT	19 (100.0)	8 (66.7)	0.007	n.a.
		TT	0 (0.0)	4 (33.3)		n.a.
	Recessive model of C	CC	13 (68.4)	3 (25.0)	0.024	1
		CT+TT	6 (31.6)	9 (75.0)		6.500 [1.28-33.03]
MPO	Dominant model of G	GG+GA	69 (93.2)	41 (91.1)	0.670	n.a.
		AA	5 (6.8)	4 (8.9)		n.a.
	Recessive model of G	GG	35 (47.3)	21 (46.7)	0.947	n.a.
		GA+AA	39 (52.7)	24 (53.3)		n.a.
CAT	Dominant model of C	CC+CT	65 (95.6)	37 (100.0)	0.195	n.a.
		TT	3 (4.4)	0 (0.0)		n.a.
	Recessive model of C	CC	57 (83.8)	30 (81.1)	0.722	n.a.
		CT+TT	11 (16.2)	7 (18.9)		n.a.

Chi-square test. AQP3, Aquaporin-3; AQP7, Aquaporin-7; NOS3, Endothelial Nitric Oxide Synthase; CYBA, Cytochrome B-245 Alpha Chain; MPO, Myeloperoxidase; CAT, Catalase; n.a., not applicable.

Association between hematological, biochemical and anthropological parameters and oxidative stress-related genes

As previously described, our findings showed that carriers of A allele of AQP3 genetic polymorphism were more frequent in postpregnancy hypertensive women when compared to CC or AC+CC genotypes according to each model of distribution (Table

3.4). In addition, a higher systolic and diastolic pressure were also observed in women carrying the AA+AC genotype of AQP3 polymorphism: systolic (mmHg; 134.67 ± 18.913 vs 125.09 ± 14.398 ; $p=0.017$) and diastolic (mmHg); 88.12 ± 18.594 vs 77.09 ± 16.099 ; $p=0.008$), compared to NBPPP (Table 3.5).

Although no statistical differences in genotype frequencies of AQP7 polymorphism between PE and NBPP before pregnancy and HT and NBPPP after pregnancy were detected, higher levels of non-HDL cholesterol were more associated with CT+TT (160.29 ± 33.606 mg dL⁻¹) of that polymorphism compared to CC (143.67 ± 29.802 mg dL⁻¹) ($p=0.037$) (Table 3. 6).

During pregnancy, genotypes BB+BA of NOS polymorphism were more frequently found in PE than in NT. This genotype was also associated with a decreased RDW (%) (12.88 ± 0.78) compared to AA genotype (15.24 ± 6.44) ($p<0.001$) (Table 3.7). On the other hand, although not associated with PE predisposition, CT+TT genotypes of CYBA genetic polymorphism were more frequently found in hypertensive women in relation to normotensive women after pregnancy, and this genotype was also associated with several cardiovascular risk biomarkers.

CT+TT genotypes of CYBA genetic polymorphism were more associated with increased weight (Kg) (CT+TT: 73.88 ± 14.58 vs CC: 63.10 ± 7.70 ; $p=0.014$) higher waist circumference (cm) (CT+TT: 91.03 ± 14.00 vs CC: 79.67 ± 7.72 ; $p=0.010$) and hip (cm) (CT+TT: 107.60 ± 7.05 vs CC: 99.13 ± 8.97 , $p=0.008$). Systolic (mmHg) (CT+TT: 141.07 ± 13.39 vs CC: 122.19 ± 12.14 ; $p<0.001$) and diastolic blood pressure (mmHg) (CT+TT: 92.07 ± 14.21 vs CC: 71.69 ± 12.21 , $p<0.001$) also demonstrated an increase in CT+TT genotypes of that genetic polymorphism (Table 3.8).

No significant differences in hematological, biochemical and anthropological parameters were observed in genotype distribution of dominant and recessive models of MPO and CAT in this population (Table 3.9 and 10, respectively).

Table 3.5. Association between hematological, biochemical and anthropological parameters and rs2231231 genotypes (dominant and recessive models) of AQP3 gene

Characteristics	AQP3					
	Dominant model of A			Recessive model of A		
	AA+AC	CC	p value	AA	AC+CC	p value
Actual Age (years)	44 (35.50 ± 6.08)	36 (34.61 ± 5.37)	0.495	11 (34.36 ± 4.61)	69 (35.22 ± 5.94)	0.651
Systolic (mmHg)	43 (134.67 ± 18.91)	34 (125.09 ± 14.40)	0.017	11 (138.91 ± 13.82)	66 (129.03 ± 17.88)	0.075
Diastolic (mmHg)	43 (88.12 ± 18.59)	34 (77.09 ± 16.10)	0.008	11 (92.45 ± 16.39)	66 (81.71 ± 18.23)	0.081
Pulse pressure	43 (46.56 ± 9.99)	34 (48.00 ± 11.90)	0.565	11 (46.45 ± 11.99)	66 (47.32 ± 10.72)	0.808
Weight (kg)	44 (69.76 ± 10.76)	36 (68.92 ± 13.55)	0.823	11 (73.36 ± 14.52)	69 (68.60 ± 11.56)	0.225
WC (cm)	42 (86.67 ± 12.25)	35 (87.59 ± 11.64)	0.738	11 (88.45 ± 11.91)	66 (86.86 ± 11.98)	0.683
HC (cm)	42 (103.75 ± 9.27)	35 (103.84 ± 10.75)	0.968	11 (107.36 ± 7.41)	66 (103.20 ± 10.18)	0.198
CRP (mg/L)	40 (0.68 ± 0.506)	34 (0.66 ± 0.553)	0.886	11 (0.618 ± 0.500)	63 (0.6841 ± 0.532)	0.703
MPO (ng/mL)	16 (55.34 ± 29.54)	18 (69.22 ± 50.83)	0.332	n.a.	34 (62.69 ± 42.16)	n.a.
Nitrites (μmol/L)	14 (17.06 ± 5.54)	13 (15.38 ± 5.32)	0.438	n.a.	27 (16.25 ± 5.40)	n.a.
NO _x (μmol/L)	14 (99.26 ± 41.37)	12 (98.44 ± 49.13)	0.964	n.a.	26 (98.89 ± 44.18)	n.a.
AST (UI/L)	44 (19.95 ± 7.79)	37 (20.84 ± 6.62)	0.588	11 (18.45 ± 3.59)	70 (20.66 ± 7.64)	0.352
ALT (UI/L)	44 (22.16 ± 14.16)	37 (21.78 ± 11.85)	0.899	11 (21.82 ± 10.49)	70 (22.01 ± 13.50)	0.963
t-Cholesterol (mg/dL)	44 (198.09 ± 30.56)	37 (207.00 ± 36.58)	0.236	11 (195.27 ± 16.04)	70 (203.24 ± 35.46)	0.225
Non-HDL Cholesterol(mg/dL)	44 (151.00 ± 28.47)	36 (158.8 ± 38.45)	0.297	11 (146.73 ± 18.14)	69 (155.78 ± 35.10)	0.202
LDL (mg/dL)	44 (132.56 ± 25.96)	36 (137.68 ± 40.11)	0.493	11 (127.85 ± 13.53)	69 (135.99 ± 35.01)	0.174
HDL (mg/dL)	44 (47.09 ± 7.84)	36 (48.69 ± 8.78)	0.391	11 (48.55 ± 9.19)	69 (47.70 ± 8.17)	0.754
Apo A (mg/dL)	42 (0.96 ± 0.16)	36 (0.99 ± 0.19)	0.441	10 (0.97 ± 0.15)	68 (0.97 ± 0.18)	0.966
Apo B (mg/dL)	42 (0.60 ± 0.14)	36 (0.64 ± 0.14)	0.193	10 (0.58 ± 0.12)	68 (0.63 ± 0.14)	0.304
Glucose (mg/dL)	44 (86.07 ± 10.41)	37 (85.43 ± 7.41)	0.757	11 (79.27 ± 9.05)	70 (86.80 ± 8.76)	0.01
TAG (mg/dL)	44 (92.18 ± 36.81)	37 (104.62 ± 62.34)	0.269	11 (94.36 ± 54.74)	70 (98.41 ± 49.80)	0.805
RDW%	44 (12.88 ± 0.87)	36 (12.8 ± 0.65)	0.677	11 (12.37 ± 0.50)	69 (12.93 ± 0.79)	0.028
Total Proteins	44 (7.33 ± 0.45)	36 (7.27 ± 0.36)	0.534	11 (7.57 ± 0.57)	69 (7.26 ± 0.37)	0.099
MCHC (g/dL)	44 (32.42 ± 0.67)	37 (32.66 ± 0.61)	0.107	11 (32.75 ± 0.65)	70 (32.50 ± 0.67)	0.255

Independent samples t-student. The values referred to absolute frequency (average ± standard deviation). WC, waist circumference; HC, hip circumference; CRP, C reactive protein; MPO, Myeloperoxidase; AST, aspartate transaminase; ALT, alanine transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, Apolipoprotein; TAG, triacylglycerols; RDW, Red cell distribution width; MCHC, mean corpuscular hemoglobin concentration.

Table 3.6. Association between hematological, biochemical and anthropological parameters and rs2989924 genotypes (dominant and recessive models) of AQP7 gene

Characteristics	AQP7					
	Dominant model of C			Recessive model of C		
	CC+CT	TT	p value	CC	CT+TT	p value
Actual Age (years)	64 (34.56 ± 6.15)	22 (36.82 ± 5.08)	0.126	23 (32.74 ± 5.74)	63 (36.02 ± 5.82)	0.023
Systolic (mmHg)	62 (127.21 ± 13.70)	21 (134.14 ± 26.60)	0.264	23 (124.00 ± 14.05)	60 (130.87 ± 18.97)	0.119
Diastolic (mmHg)	62 (80.39 ± 14.39)	21 (87.24 ± 24.55)	0.238	23 (79.04 ± 13.83)	60 (83.30 ± 18.23)	0.327
Pulse pressure	62 (46.82 ± 10.75)	21 (46.90 ± 10.94)	0.976	23 (44.96 ± 10.62)	60 (47.57 ± 10.78)	0.324
Weight (kg)	64 (67.12 ± 10.69)	22 (69.30 ± 11.30)	0.419	23 (67.56 ± 13.32)	63 (67.72 ± 9.88)	0.950
WC (cm)	62 (85.63 ± 12.09)	21 (91.17 ± 21.89)	0.151	23 (103.24 ± 9.71)	60 (102.93 ± 9.11)	0.526
HC (cm)	62 (102.61 ± 9.96)	21 (104.19 ± 6.66)	0.417	23 (26.91 ± 5.77)	60 (29.14 ± 5.98)	0.891
CRP (mg/L)	59 (0.58 ± 0.43)	21 (0.64 ± 0.52)	0.592	23 (0.47 ± 0.36)	57 (0.64 ± 0.48)	0.109
MPO (ng/mL)	28 (68.53 ± 41.92)	8 (44.10 ± 32.89)	0.139	8 (65.95 ± 34.42)	28 (62.28 ± 43.19)	0.827
Nitrites (μmol/L)	24 (15.85 ± 5.44)	4 (29.15 ± 15.76)	0.109	7 (15.34 ± 6.60)	21 (18.56 ± 9.26)	0.406
NO _x (μmol/L)	23 (100.93 ± 42.24)	4 (80.20 ± 25.85)	0.355	7 (82.47 ± 24.01)	20 (103.25 ± 44.11)	0.137
AST (UI/L)	65 (19.74 ± 7.17)	22 (20.55 ± 7.20)	0.650	24 (19.54 ± 9.55)	63 (20.10 ± 6.07)	0.749
ALT (UI/L)	65 (21.37 ± 12.14)	22 (22.91 ± 14.55)	0.628	24 (21.08 ± 16.98)	63 (22.02 ± 10.92)	0.763
t-Cholesterol (mg/dL)	65 (201.74 ± 33.60)	22 (209.00 ± 33.44)	0.383	24 (193.33 ± 24.47)	63 (207.48 ± 34.96)	0.078
Non-HDL Cholesterol(mg/dL)	65 (153.11 ± 33.60)	21 (163.52 ± 31.72)	0.214	24 (143.67 ± 29.80)	62 (160.29 ± 33.61)	0.037
LDL (mg/dL)	65 (134.31 ± 33.44)	21 (141.27 ± 31.32)	0.403	24 (126.54 ± 28.41)	62 (139.67 ± 33.98)	0.097
HDL (mg/dL)	65 (48.63 ± 8.52)	21 (46.52 ± 7.40)	0.131	24 (49.67 ± 8.64)	62 (47.52 ± 8.11)	0.282
Apo A (mg/dL)	61 (0.98 ± 0.18)	22 (0.95 ± 0.16)	0.415	22 (0.97 ± 0.16)	61 (0.97 ± 0.18)	0.917
Apo B (mg/dL)	61 (0.62 ± 0.14)	22 (0.64 ± 0.15)	0.488	22 (0.58 ± 0.13)	61 (0.64 ± 0.14)	0.098
Glucose (mg/dL)	65 (84.55 ± 9.58)	22 (83.77 ± 9.22)	0.739	24 (81.96 ± 10.45)	63 (85.27 ± 8.95)	0.145
TAG (mg/dL)	65 (93.98 ± 6.96)	22 (108.91 ± 75.13)	0.222	24 (85.63 ± 40.12)	63 (102.38 ± 52.02)	0.158
RDW%	65 (13.06 ± 2.41)	21 (13.08 ± 0.64)	0.971	24 (13.58 ± 3.85)	62 (12.87 ± 0.69)	0.377
Total Proteins	65 (7.25 ± 0.38)	21 (7.49 ± 0.46)	0.022	24 (7.25 ± 0.426)	62 (7.33 ± 0.41)	0.417
MCHC (g/dL)	65 (32.52 ± 0.63)	22 (32.63 ± 0.77)	0.510	24 (32.4 ± 0.71)	63 (32.59 ± 0.64)	0.329

Independent samples t-student. The values referred to absolute frequency (average ± standard deviation). WC, waist circumference; HC, hip circumference; CRP, C reactive protein; MPO, Myeloperoxidase; AST, aspartate transaminase; ALT, alanine transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, Apolipoprotein; TAG, triacylglycerols; RDW, Red cell distribution width; MCHC, mean corpuscular hemoglobin concentration.

Table 3.7. Association between hematological, biochemical and anthropological parameters and VNTR intron 4 genotypes (dominant and recessive models) of NOS3 gene

Characteristics	NOS3					
	Dominant model of B			Recessive model of B		
	BB+BA	AA	<i>p</i> value	BB	BA+AA	<i>p</i> value
Actual Age (years)	135 (35.16 ± 5.55)	8 (34.88 ± 4.67)	0.886	102 (35.17 ± 5.50)	41 (35.10 ± 5.54)	0.946
Systolic (mmHg)	132 (129.82 ± 16.99)	8 (120.75 ± 17.71)	0.146	100 (139.94 ± 17.53)	40 (127.70 ± 16.05)	0.486
Diastolic (mmHg)	132 (81.59 ± 17.13)	8 (70.13 ± 11.34)	0.064	100 (81.54 ± 17.45)	40 (79.43 ± 16.05)	0.509
Pulse pressure	132 (18.23 ± 10.92)	8 (50.63 ± 14.59)	0.555	100 (48.40 ± 11.40)	40 (48.28 ± 10.48)	0.952
Weight (kg)	134 (67.62 ± 11.01)	8 (74.28 ± 19.823)	0.118	101 (68.00 ± 11.18)	41 (67.99 ± 12.95)	0.995
WC (cm)	131 (86.68 ± 14.20)	7 (90.14 ± 15.88)	0.533	98 (87.46 ± 15.07)	40 (85.38 ± 12.04)	0.436
HC (cm)	131 (103.19 ± 9.88)	7 (105.43 ± 16.60)	0.735	98 (102.96 ± 10.26)	40 (104.14 ± 10.30)	0.542
CRP (mg/L)	126 (0.67 ± 0.73)	8 (0.76 ± 0.59)	0.710	95 (0.691 ± 0.82)	39 (0.623 ± 0.38)	0.516
MPO (ng/mL)	64 (63.045 ± 40.16)	6 (76.07 ± 49.75)	0.459	49 (62.75 ± 40.99)	21 (67.466 ± 41.18)	0.661
Nitrites (µmol/L)	51 (15.47 ± 7.53)	6 (12.70 ± 5.21)	0.387	41 (14.98 ± 7.82)	16 (15.681 ± 6.11)	0.747
NO _x (µmol/L)	48 (88.81 ± 38.98)	6 (100.10 ± 48.76)	0.518	41 (86.88 ± 40.65)	13 (100.100 ± 36.70)	0.301
AST (UI/L)	136 (19.62 ± 6.36)	8 (22.75 ± 4.40)	0.173	103 (19.53 ± 5.59)	41 (20.44 ± 7.86)	0.438
ALT (UI/L)	136 (20.65 ± 11.52)	8 (22.50 ± 13.09)	0.663	103 (20.38 ± 10.24)	41 (21.71 ± 14.48)	0.536
t-Cholesterol (mg/dL)	136 (207.93 ± 38.04)	8 (194.88 ± 16.58)	0.337	103 (206.03 ± 37.78)	41 (210.17 ± 36.21)	0.549
Non-HDL Cholesterol (mg/dL)	135 (158.56 ± 37.63)	8 (142.63 ± 18.54)	0.237	103 (156.56 ± 37.13)	40 (160.60 ± 36.88)	0.560
LDL (mg/dL)	135 (138.65 ± 37.03)	8 (122.25 ± 37.03)	0.015	103 (137.36 ± 36.85)	40 (138.70 ± 35.30)	0.844
HDL (mg/dL)	135 (49.50 ± 8.43)	8 (122.25 ± 14.02)	0.370	13 (49.47 ± 8.21)	40 (50.15 ± 8.91)	0.663
Apo A (mg/dL)	131 (0.98 ± 0.19)	8 (1.05 ± 0.13)	0.283	99 (0.96 ± 0.17)	40 (1.033 ± 0.21)	0.058
Apo B (mg/dL)	131 (0.63 ± 0.14)	8 (0.62 ± 0.09)	0.948	99 (0.62 ± 0.14)	40 (0.631 ± 0.14)	0.717
Glucose (mg/dL)	136 (84.19 ± 9.44)	8 (83.00 ± 8.35)	0.728	103 (84.15 ± 9.36)	41 (84.07 ± 9.48)	0.967
TAG (mg/dL)	136 (99.37 ± 48.74)	8 (101.88 ± 59.02)	0.889	103 (96.02 ± 46.21)	41 (108.27 ± 55.45)	0.178
RDW%	134 (12.88 ± 0.78)	8 (15.24 ± 6.45)	<0.001	102 (12.85 ± 0.74)	40 (13.423 ± 2.99)	0.240
Total Proteins	135 (7.28 ± 0.41)	8 (7.01 ± 0.11)	0.335	103 (7.28 ± 0.44)	40 (7.218 ± 0.33)	0.350
MCHC (g/dL)	136 (32.53 ± 0.62)	8 (32.81 ± 0.48)	0.204	103 (32.52 ± 0.63)	41 (32.600 ± 0.58)	0.485

Independent samples t-student. The values referred to absolute frequency (average ± standard deviation). WC, waist circumference; HC, hip circumference; CRP, C reactive protein; MPO, Myeloperoxidase; AST, aspartate transaminase; ALT, alanine transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, Apolipoprotein; TAG, triacylglycerols; RDW, Red cell distribution width; MCHC, mean corpuscular hemoglobin concentration.

Table 3.8. Association between hematological, biochemical and anthropological parameters and rs4673 genotypes (dominant and recessive models) of CYBA gene

Characteristics	CYBA					
	Dominant model of C			Recessive model of C		
	CC+CT	TT	p value	CC	CT+TT	p value
Actual Age (years)	28 (33.07 ± 5.60)	4 (41.00 ± 1.63)	0.495	16 (31.94 ± 5.03)	16 (36.19 ± 6.06)	0.039
Systolic (mmHg)	27 (127.30 ± 11.86)	4 (158.50 ± 12.01)	0.017	16 (122.19 ± 12.14)	15 (141.07 ± 13.39)	<0.001
Diastolic (mmHg)	27 (77.85 ± 13.78)	4 (106.50 ± 12.66)	0.008	16 (71.69 ± 12.21)	15 (92.07 ± 14.21)	<0.001
Pulse pressure	27 (49.44 ± 11.65)	4 (52.00 ± 9.83)	0.565	16 (50.50 ± 11.28)	15 (49.00 ± 11.71)	0.719
Weight (kg)	28 (67.99 ± 12.37)	4 (72.00 ± 16.673)	0.0564	16 (63.10 ± 7.70)	16 (73.88 ± 14.58)	0.014
WC (cm)	26 (84.81 ± 12.65)	4 (88.88 ± 12.76)	0.738	15 (79.67 ± 7.72)	15 (91.03 ± 14.00)	0.010
HC (cm)	26 (102.88 ± 9.21)	4 (106.50 ± 8.06)	0.968	15 (99.13 ± 8.97)	15 (107.60 ± 7.05)	0.008
CRP (mg/L)	27 (0.66 ± 0.41)	4 (0.850 ± 0.74)	0.431	16 (0.57 ± 0.33)	15 (0.80 ± 0.54)	0.158
MPO (ng/mL)	14 (75.52 ± 39.40)	n.a.	n.a.	9 (79.90 ± 43.10)	5 (67.64 ± 34.77)	0.597
Nitrites (µmol/L)	13 (16.99 ± 6.12)	n.a.	n.a.	8 (16.21 ± 5.79)	5 (18.22 ± 7.10)	0.587
NO _x (µmol/L)	13 (83.97 ± 35.79)	n.a.	n.a.	8 (84.38 ± 35.39)	5 (83.32 ± 40.62)	0.961
AST (UI/L)	28 (21.18 ± 9.04)	4 (19.50 ± 3.79)	0.719	16 (21.13 ± 5.01)	16 (20.81 ± 11.20)	0.919
ALT (UI/L)	28 (21.89 ± 15.87)	4 (16.50 ± 7.05)	0.513	16 (20.13 ± 9.56)	16 (22.31 ± 19.40)	0.689
t-Cholesterol (mg/dL)	28 (197.43 ± 34.93)	4 (204.00 ± 29.77)	0.724	16 (192.63 ± 27.49)	16 (203.88 ± 39.50)	0.357
Non-HDL Cholesterol (mg/dL)	28 (149.07 ± 36.27)	4 (155.50 ± 27.96)	0.737	16 (141.56 ± 29.07)	16 (158.19 ± 39.27)	0.184
LDL (mg/dL)	28 (128.71 ± 37.56)	4 (135.45 ± 39.57)	0.734	16 (120.53 ± 30.64)	16 (138.58 ± 40.15)	0.163
HDL (mg/dL)	28 (48.36 ± 7.98)	4 (48.50 ± 5.45)	0.973	16 (51.06 ± 7.20)	16 (45.69 ± 7.31)	0.045
Apo A (mg/dL)	28 (0.97 ± 0.18)	4 (1.11 ± 0.18)	0.168	16 (0.97 ± 0.16)	16 (1.02 ± 0.20)	0.439
Apo B (mg/dL)	28 (0.61 ± 0.12)	4 (0.64 ± 0.06)	0.651	16 (0.60 ± 0.11)	16 (0.63 ± 0.12)	0.525
Glucose (mg/dL)	28 (84.75 ± 9.05)	4 (84.50 ± 6.66)	0.958	16 (84.81 ± 7.13)	16 (84.63 ± 10.27)	0.953
TAG (mg/dL)	28 (101.82 ± 62.64)	4 (100.25 ± 32.64)	0.962	16 (105.19 ± 79.97)	16 (98.06 ± 30.32)	0.741
RDW%	28 (13.00 ± 0.92)	4 (12.88 ± 0.66)	0.791	16 (12.88 ± 0.64)	16 (13.10 ± 1.09)	0.506
Total Proteins	28 (7.24 ± 0.33)	4 (7.28 ± 0.53)	0.836	16 (7.18 ± 0.22)	16 (7.30 ± 0.44)	0.343
MCHC (g/dL)	28 (32.50 ± 0.67)	4 (32.50 ± 0.36)	0.992	16 (32.53 ± 0.59)	16 (32.48 ± 0.69)	0.806

Independent samples t-student. The values referred to absolute frequency (average ± standard deviation). waist circumference; HC, hip circumference; CRP, C reactive protein; MPO, Myeloperoxidase; AST, aspartate transaminase; ALT, alanine transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, Apolipoprotein; TAG, triacylglycerols; RDW, Red cell distribution width; MCHC, mean corpuscular hemoglobin concentration.

Table 3.9. Association between hematological, biochemical and anthropological parameters and rs2333227 genotypes (dominant and recessive models) of MPO gene

Characteristics	MPO					
	Dominant model of G			Recessive model of G		
	GG+GA	AA	<i>p</i> value	GG	GA+AA	<i>p</i> value
Actual Age (years)	113 (34.86 ± 5.54)	9 (36.89 ± 4.05)	0.285	58 (34.98 ± 5.87)	64 (35.03 ± 5.10)	0.961
Systolic (mmHg)	110 (129.65 ± 17.67)	9 (136.44 ± 15.57)	0.266	55 (129.20 ± 16.39)	64 (130.98 ± 18.59)	0.583
Diastolic (mmHg)	110 (81.22 ± 17.09)	9 (90.33 ± 16.93)	0.126	55 (79.67 ± 15.33)	64 (83.83 ± 18.52)	0.189
Pulse pressure	110 (48.43 ± 11.42)	9 (46.11 ± 10.51)	0.558	55 (49.53 ± 11.56)	64 (47.16 ± 11.10)	0.257
Weight (kg)	113 (67.96 ± 12.23)	9 (66.89 ± 7.27)	0.796	59 (66.31 ± 10.91)	63 (69.36 ± 12.69)	0.158
WC (cm)	109 (86.69 ± 14.92)	9 (88.44 ± 10.10)	0.730	56 (86.59 ± 16.77)	62 (87.03 ± 12.41)	0.870
HC (cm)	109 (104.00 ± 9.66)	9 (104.89 ± 10.36)	0.793	56 (103.04 ± 9.25)	62 (105.01 ± 10.02)	0.271
CRP (mg/L)	104 (0.72 ± 0.76)	9 (0.52 ± 0.35)	0.427	52 (0.61 ± 0.45)	61 (0.80 ± 0.91)	0.182
MPO (ng/mL)	55 (66.52 ± 36.30)	4 (26.67 ± 20.52)	0.035	29 (66.67 ± 41.67)	30 (61.06 ± 31.77)	0.562
Nitrites (μmol/L)	44 (15.89 ± 7.94)	3 (10.97 ± 3.41)	0.296	23 (17.54 ± 9.74)	24 (13.69 ± 4.85)	0.091
NO _x (μmol/L)	41 (89.12 ± 38.10)	3 (81.93 ± 37.05)	0.754	21 (91.41 ± 39.80)	23 (86.10 ± 36.28)	0.646
AST (UI/L)	113 (19.96 ± 6.61)	9 (20.00 ± 4.61)	0.987	58 (19.76 ± 7.40)	64 (20.16 ± 5.56)	0.736
ALT (UI/L)	113 (21.51 ± 12.55)	9 (16.67 ± 5.00)	0.254	58 (22.28 ± 13.15)	64 (20.14 ± 11.30)	0.337
t-Cholesterol (mg/dL)	113 (204.81 ± 35.85)	9 (206.11 ± 50.10)	0.919	58 (209.89 ± 37.73)	64 (200.42 ± 35.67)	0.158
Non-HDL Cholesterol (mg/dL)	112 (155.65 ± 36.17)	9 (153.78 ± 46.02)	0.884	58 (158.98 ± 38.28)	63 (152.32 ± 35.35)	0.321
LDL (mg/dL)	112 (136.29 ± 37.05)	9 (132.44 ± 38.00)	0.765	58 (139.92 ± 39.18)	63 (132.40 ± 34.74)	0.265
HDL (mg/dL)	112 (49.32 ± 8.69)	9 (52.33 ± 6.80)	0.313	58 (50.88 ± 8.29)	63 (48.32 ± 8.72)	0.101
Apo A (mg/dL)	110 (0.97 ± 0.18)	9 (1.02 ± 0.18)	0.401	55 (1.01 ± 0.16)	64 (0.95 ± 0.19)	0.085
Apo B (mg/dL)	110 (0.62 ± 0.14)	9 (0.63 ± 0.17)	0.852	55 (0.63 ± 0.13)	64 (0.60 ± 0.14)	0.215
Glucose (mg/dL)	113 (84.30 ± 9.57)	9 (86.56 ± 7.88)	0.493	58 (84.14 ± 9.87)	64 (84.77 ± 9.12)	0.716
TAG (mg/dL)	113 (96.47 ± 49.47)	9 (106.67 ± 49.40)	0.553	58 (95.29 ± 45.13)	64 (98.97 ± 53.15)	0.683
RDW%	112 (13.07 ± 1.91)	9 (12.34 ± 0.45)	0.257	58 (12.97 ± 0.96)	63 (13.07 ± 2.40)	0.765
Total Proteins	112 (7.29 ± 0.42)	9 (7.22 ± 0.38)	0.645	58 (7.22 ± 0.38)	63 (7.34 ± 0.44)	0.129
MCHC (g/dL)	113 (32.53 ± 0.63)	9 (32.93 ± 0.51)	0.066	58 (32.51 ± 0.61)	64 (32.61 ± 0.65)	0.420

Independent samples t-student. The values referred to absolute frequency (average ± standard deviation). WC, waist circumference; HC, hip circumference; CRP, C reactive protein; MPO, Myeloperoxidase; AST, aspartate transaminase; ALT, alanine transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, Apolipoprotein; TAG, triacylglycerols; RDW, Red cell distribution width; MCHC, mean corpuscular hemoglobin concentration.

Table 3.10. Association between hematological, biochemical and anthropological parameters and rs1001179 genotypes (dominant and recessive models) of CAT gene

Characteristics	CAT					
	Dominant model of C			Recessive model of C		
	CC+CT	TT	p value	CC	CT+TT	p value
Actual Age (years)	105 (35.05 ± 5.70)	3 (128.00 ± 9.17)	0.542	90 (35.17 ± 5.54)	18 (34.11 ± 6.57)	0.476
Systolic (mmHg)	102 (130.30 ± 17.28)	3 (128.00 ± 9.17)	0.819	87 (130.56 ± 17.72)	18 (128.67 ± 13.85)	0.670
Diastolic (mmHg)	102 (81.90 ± 17.29)	3 (78.67 ± 2.31)	0.748	87 (81.69 ± 17.07)	18 (82.39 ± 17.43)	0.875
Pulse pressure	102 (48.40 ± 11.57)	3 (49.33 ± 7.02)	0.890	87 (48.87 ± 10.87)	18 (46.28 ± 14.07)	0.384
Weight (kg)	104 (68.21 ± 11.95)	3 (62.33 ± 11.02)	0.402	89 (67.93 ± 11.59)	18 (68.63 ± 13.77)	0.821
WC (cm)	103 (87.01 ± 15.04)	3 (87.33 ± 15.18)	0.971	88 (86.80 ± 15.31)	18 (88.11 ± 13.57)	0.736
HC (cm)	103 (104.07 ± 9.79)	3 (96.00 ± 11.14)	0.164	88 (104.16 ± 9.80)	18 (102.28 ± 10.35)	0.464
CRP (mg/L)	96 (0.72 ± 0.79)	3 (0.97 ± 0.93)	0.593	81 (0.69 ± 0.83)	18 (0.87 ± 0.56)	0.403
MPO (ng/mL)	49 (68.28 ± 40.04)	3 (42.07 ± 16.56)	0.268	43 (65.98 ± 38.32)	9 (70.56 ± 46.93)	0.755
Nitrites (µmol/L)	40 (15.36 ± 8.34)	3 (16.40 ± 3.97)	0.832	34 (15.13 ± 8.75)	9 (16.57 ± 5.10)	0.640
NO _x (µmol/L)	40 (85.46 ± 39.01)	3 (129.20 ± 14.87)	0.063	34 (87.71 ± 41.36)	9 (91.51 ± 32.74)	0.800
AST (UI/L)	106 (19.77 ± 6.29)	3 (15.67 ± 3.22)	0.264	91 (19.62 ± 6.51)	18 (19.89 ± 4.95)	0.866
ALT (UI/L)	106 (20.08 ± 10.51)	3 (17.33 ± 3.06)	0.653	91 (19.68 ± 10.60)	18 (21.67 ± 9.28)	0.461
t-Cholesterol (mg/dL)	106 (204.92 ± 37.11)	3 (192.67 ± 7.10)	0.575	91 (206.32 ± 37.00)	18 (195.78 ± 37.13)	0.272
Non-HDL Cholesterol (mg/dL)	105 (155.37 ± 37.54)	3 (145.67 ± 10.07)	0.657	90 (156.38 ± 37.73)	18 (148.72 ± 33.81)	0.426
LDL (mg/dL)	105 (135.26 ± 37.52)	3 (125.47 ± 19.34)	0.655	90 (136.09 ± 38.15)	18 (129.46 ± 31.86)	0.491
HDL (mg/dL)	105 (49.71 ± 8.78)	3 (47.00 ± 3.00)	0.252	90 (50.16 ± 8.69)	18 (47.06 ± 8.38)	0.168
Apo A (mg/dL)	102 (0.98 ± 0.18)	3 (0.96 ± 0.15)	0.846	88 (0.98 ± 0.17)	17 (0.95 ± 0.23)	0.472
Apo B (mg/dL)	102 (0.62 ± 0.14)	3 (0.68 ± 0.11)	0.473	88 (0.62 ± 0.14)	17 (0.61 ± 0.14)	0.736
Glucose (mg/dL)	106 (84.65 ± 9.26)	3 (88.33 ± 7.51)	0.497	91 (84.63 ± 9.31)	18 (85.39 ± 8.88)	0.750
TAG (mg/dL)	106 (100.18 ± 2.10)	3 (101.00 ± 46.87)	0.979	91 (100.97 ± 53.37)	18 (96.33 ± 43.91)	0.730
RDW%	104 (13.09 ± 1.97)	3 (12.40 ± 0.35)	0.549	89 (13.14 ± 2.07)	18 (12.72 ± 1.10)	0.413
Total Proteins	105 (7.31 ± 0.42)	3 (7.13 ± 0.06)	0.486	90 (7.28 ± 0.41)	18 (7.40 ± 0.479)	0.268
MCHC (g/dL)	106 (32.56 ± 0.63)	3 (32.53 ± 0.15)	0.939	91 (32.56 ± 0.58)	18 (32.57 ± 0.81)	0.964

Independent samples t-student. The values referred to absolute frequency (average ± standard deviation). WC, waist circumference; HC, hip circumference; CRP, C reactive protein; MPO, Myeloperoxidase; AST, aspartate transaminase; ALT, alanine transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, Apolipoprotein; TAG, triacylglycerols; RDW, Red cell distribution width; MCHC, mean corpuscular hemoglobin concentration.

AQP3 and CYBA polymorphisms correlate with higher susceptibility to cardiovascular risk in women with previous preeclampsia

The previous groups, NBPP vs PE during pregnancy and NBPPP vs HT after pregnancy, were then reclassified.

Table 3.11. Distribution of the phenotypes of oxidative stress-associated genes in women with normal blood pressure in pregnancy (NBPP) and preeclamptic women (PE); and in women with normal blood pressure postpregnancy (NBPPP) and hypertensive women (HT).

		Genotype	PE>HT n=27 n(%)	PE>NBPPP n=27 n(%)	NBPP>HT n=3 n(%)	NBPP>NBPPP n=20 n(%)	p value
AQP3	Dominant model of A	AA+AC	19 (70.4)	14 (51.9)	3 (100.0)	7 (35)	0.039
		CC	8 (29.6)	13 (48.1)	0 (0.0)	13 (65)	
	Recessive model of A	AA	7 (25.9)	2 (7.4)	1 (33.3)	1 (5)	0.097
		AC+CC	20 (74.1)	25 (92.6)	2 (66.7)	19 (95)	
AQP7	Dominant model of C	CC+CT	18 (66.7)	24 (80.0)	2 (66.7)	18 (78.3)	0.654
		TT	9 (33.3)	6 (20.0)	1 (33.3)	5 (21.7)	
	Recessive model of C	CC	6 (22.2)	8 (26.7)	1 (33.3)	8 (34.8)	0.791
		CT+TT	21 (77.8)	22 (73.3)	2 (66.7)	15 (65.2)	
NOS3	Dominant model of B	BB+BA	42 (100.0)	44 (95.7)	5 (83.3)	41 (89.1)	0.097
		AA	0 (0.0)	2 (4.3)	1 (16.7)	5 (10.9)	
	Recessive model of B	BB	33 (78.6)	34 (73.9)	5 (83.3)	28 (60.9)	0.249
		BA+AA	9 (21.4)	12 (86.1)	1 (16.7)	18 (39.1)	
CYBA	Dominant model of C	CC+CT	8 (66.7)	14 (100.0)	0 (0.0)	5 (100.0)	0.026
		TT	4 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	
	Recessive model of C	CC	3 (25.0)	9 (64.3)	0 (0.0)	4 (80.0)	0.052
		CT+TT	9 (75.0)	5 (35.7)	0 (0.0)	1 (20.0)	
MPO	Dominant model of G	GG+GA	35 (89.7)	36 (97.3)	6 (100.0)	33 (89.2)	0.440
		AA	4 (10.3)	1 (2.7)	0 (0.0)	4 (10.8)	
	Recessive model of G	GG	20 (51.3)	20 (54.1)	1 (16.7)	15 (40.5)	0.277
		GA+AA	19 (48.7)	17 (45.9)	5 (83.3)	22 (59.5)	
CAT	Dominant model of C	CC+CT	31 (100.0)	33 (91.7)	6 (100.0)	32 (100.0)	0.116
		TT	0 (0.0)	3 (8.3)	0 (0.0)	0 (0.0)	
	Recessive model of C	CC	24 (77.4)	28 (77.8)	6 (100.0)	29 (90.6)	0.272
		CT+TT	7 (2.6)	8 (22.2)	0 (0.0)	3 (9.4)	

Chi-square test. PE>HT, Group1; PE>NBPPP, Group 2; NBPP>HT, Group3; NBPP>NBPPP, Group 4; AQP3, Aquaporin-3; AQP7, Aquaporin-7; NOS3, Endothelial Nitric Oxide Synthase; CYBA, Cytochrome B-245 Alpha Chain; MPO, Myeloperoxidase; CAT, Catalase.

A previous publication from our research group characterized these women and found that 47.7% of PE women developed HT (Group 1; PE>HT) while the other 53.3% became NBPPP (Group 2; PE>NBPPP) and 10.3% of NBPP women developed hypertension (Group 3; NBPP>HT) while 89.7% remained normotensive (Group 4; NBPP>NBPPP) [26].

Table 3.11 depicts the distribution of genotype frequencies according to the dominant and recessive model for each polymorphism in the 4 distinct groups.

Our data show a statistical significance for the genotypes' distribution on the dominant model of A for the AQP3 polymorphism and the 4 groups of women ($p=0.039$). In addition, an association between the genotypes' distribution of the dominant model of C for the CYBA polymorphism and the 4 groups was observed ($p=0.026$). No other statistical significances were found when evaluating the other polymorphisms, AQP7, NOS3, MPO and CAT.

Interestingly, an epistatic interaction of NOS3 BB+BA and CYBA CT+TT genotypes was associated with PE >HT group compared to PE>NBPPP group ($p=0.045$).

Discussion

Cardiovascular diseases, such as atherosclerosis, are the leading cause of death worldwide and are strongly influenced by pathologies such as obesity, hypertension, insulin resistance and dyslipidemia, which when expressed in cluster are commonly known as Metabolic Syndrome. The progression of hypertension is believed to comprise a systemic redox imbalance caused by reactive oxygen species (ROS) generation and a depressed antioxidant system that leads to oxidative stress [19].

The association between placentation-related disorders, such as preeclampsia, and the risk of developing future cardiovascular disease has been demonstrated in recent years [27]. By introducing a preventive approach into clinical practice, the relationship between cardiovascular risk/disease and obstetric complications, linked by endothelial dysfunction and oxidative stress, becomes a window of opportunity to improve women's health. Questionnaires adapted to local practices are proposed to incorporate cardiovascular and obstetrical indexes into two stages of Women's life.

In this study, a cohort of pregnant women, composed of healthy or preeclamptic subjects, was very well characterized not only during pregnancy but also from 2 to 16

years after pregnancy, stage at which a part of these women was diagnosed with hypertension. Here, apart from the evaluation of several blood-circulating cardiovascular risk biomarkers already well described in a previous publication [26], single-nucleotide polymorphisms (SNPs) for AQP3 rs2231231, AQP7 rs2989924, CYBA (p22phox) rs4673, MPO rs2333227 and CAT rs1001179 and variable number tandem repeats (VNTR) for NOS3 intron-4, were evaluated to investigate any relation between those biomarkers and the development of pre or postpregnancy hypertension.

We observed a clear association between NOS3 polymorphisms and susceptibility to preeclampsia. BB+BA polymorphism, which was associated with PE, was also associated with decreased RDW (%). Taken together, the present results suggest that the NOS3-intron-4 polymorphism seems to be a good biomarker for PE, since its correlation with this cardiovascular disease was only observed during pregnancy and not after it. A meta-analysis involving three NOS3 genetic polymorphisms (4b/a, T-786C and G894T) and PE risk showed that carriers of allele A of 4b/a suffer higher risk of PE and T-786C genetic polymorphism is associated with increased PE risk in the dominant model [28,29]. Previously, it was reported that the SNP rs1799983 (also known as Glu298Asp, E298D or G894T) was associated with an increased risk for the development of PE and further complications in a cohort of African women [30]. Another study showed that rs2070744 (also known as T786C) in the promoter of the NOS3 gene is associated with changes in markers of oxidant stress in high-risk Caucasian patients [31]. This SNP also correlates with blunted endothelium-dependent vasodilation in hypertensive patients and was associated with multivessel coronary artery disease in cross-sectional studies, but remained unsettled if it provided prognostic information about the risk of cardiovascular death [31].

Furthermore, our data represent the first association study of AQP3 genetic polymorphism and hypertension during and after pregnancy. Although no other genetic polymorphisms were associated with PE, our results also demonstrate an association between AQP3 and CYBA (p22phox) genetic polymorphisms and the development of hypertension. In addition, higher systolic and diastolic blood pressure were also observed in women carrying the AA+AC of AQP3 polymorphism. Moreover, compared to NT, women who developed HT after pregnancy exhibited a higher frequency of the allele A and thus, considered the allele of risk. Despite no previous records linking AQP3 polymorphism with hypertension have been found, making this work the first one to report such association, oxidative stress feature of hypertension as also been

described as a trigger for the development of other pathologies, such as cancer. Indeed, association analysis between rs22312331 AQP3 polymorphism with Epstein-Barr Virus-associated lymphoma and nasopharyngeal carcinoma and breast cancer in a Chinese population showed an association for AA genotype and the susceptibility for developing cancer [32,33]. Thus, this AQP3 genetic polymorphism seems to be a potential biomarker for oxidative stress and the development hypertension. Regarding the rs2989924 AQP7 polymorphism, no statistical differences between PE and NT before pregnancy and HT and NT after pregnancy were detected in the present cohort. However, higher levels of non-HDL cholesterol were associated with the CT+TT genotype compared to CC. AQP7 did not correlate with hypertension during or after pregnancy; however, it correlated with lipid profile status. Furthermore, in a previous publication, the same AQP7 SNP was evaluated in a cohort of obese and diabetic Bulgarian patients and appears to be ineffective in the pathophysiology of obesity and T2D [34]. However, another study, where rs2989924 AQP7 polymorphism (also designated by A-953G) was evaluated in a Caucasian population of diabetic patients, showed that this SNP located in the promoter of AQP7 gene can modulate the risk of obesity and associated abnormalities [35]. According to this publication, AG+GG genotype of AQP7 polymorphism correlates with T2D. Moreover, luciferase and mobility shift assays demonstrated that, compared to -953A, the -953G promoter had reduced transcriptional activity and impaired ability to bind to the transcription factor. These data strongly suggest that AQP7 downregulation is pathogenic for obesity and T2D [35].

As for CT+TT genotypes of CYBA (p22phox) genetic polymorphism, we found that it is more frequent in hypertensive women than in normotensive women after pregnancy and was also more associated with higher weight, waist and hip circumferences, and increased systolic and diastolic blood pressure.

NADPH oxidase causes vascular wall remodeling and endothelial dysfunction by generating superoxide anions (O_2^-) contributing to the initial pathogenesis of vascular diseases such as hypertension [36]. Genetic alterations in the CYBA gene, including the C242T and A640G polymorphisms, have been reported to interrupt this pathway [37]. CYBA is an essential component of NADPH oxidase. The rs4673 CYBA (also known as C242T) SNP has been associated with variation in NADPH oxidase activity in human blood vessels and therefore a decreased production of ROS and vascular oxidative stress [38]. Our data suggest that AQP3 and p22phox polymorphism may

modulate the genetic susceptibility to develop HT only after pregnancy, while NOS3 may modulate the development of preeclampsia, implying two different mechanisms in the development of PE and HT after pregnancy. On the one hand, NOS3 associated with ischemia, while AQP3 and p22phox seems to be more related to the development of essential hypertension and metabolic syndrome, though the modulation of ROS. Furthermore, an epistatic interaction of CYBA CT+TT genotype and NOS3 BB+BA genotype was associated with women that had PE and developed HT after pregnancy compared to women who had PE and after pregnancy remain normotensive.

In this study, no association between rs2333227 MPO and rs1001179 CAT polymorphisms and PE and/or HT was found. Evidences indicate that an unbalanced production of oxidized molecules within the vascular wall is one of the mechanisms that is involved in the onset and progression of cardiovascular diseases. MPO, a member of the heme peroxidase super family, has been proposed as a new risk indicator for future coronary events in healthy people and a prognostic marker in diseased patients, thus elevated serum MPO levels have been associated with a higher risk for cardiovascular disease. Previous studies have demonstrated divergent proposals about MPO SNP role in the development of cardiovascular diseases. Research on rs2333227 MPO SNP, also known as G-463A, showed that genotype -463A was associated with a decreased risk of essential hypertension and the -463A allele was associated with lower MPO expression [39,40]. Other publications describe no association between MPO and the risk of PE [41]. Contrary to MPO, CAT is one of the major antioxidant enzymes, which plays an important role in the clearance of reactive oxygen species. So far, three polymorphisms in the promoter region of the CAT have been identified and significantly associated with the activity of CAT promoter, but no association with PE or HT have been reported so far [42].

SNPs have a wide distribution and can be stably inherited, therefore, they are considered important biomarkers when they are of functional type. However, the application of studying individual SNPs is limited. The characterization of new oxidative stress-related gene SNPs, individually and in combination, seems to be a good tool to understand how susceptibility genes modulate the development of associated cardiovascular diseases.

Conclusions

In this study, NOS3 (VNTR, intron 4) and AQP3 (SNP, rs2231231) and CYBA (SNP, rs4673) functional genetic polymorphisms were found associated with the hypertensive disorders during and after pregnancy, respectively. Extended the study with a larger number of patients and combining genetic data with biochemical and anthropometric biomarkers may become a relevant approach in the prevention of cardiovascular disease.

Material and Methods

Subjects

This study was approved by the local ethics committee and informed written consent was obtained from all subjects before the collection of samples. The reported investigations were conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Design and additional results have been previously published [26]. Briefly, 150 women aged 35.24 ± 5.47 years were evaluated and 60 (40.66%) had normal blood pressure in pregnancy and 90 (59.33%) developed preeclampsia, pathologic state diagnosed at the Department of Obstetrics and Gynecology from the Júlio Diniz Maternity, Maria Pia Hospital, Oporto, Portugal. The diagnostic of preeclampsia was based on criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) [43,44]. Women of the control group, followed at the same Hospital, were invited to participate in the study according to matching age and similarity to the preeclamptic women group. Unhealthy behaviors as smoking and alcoholic habits during pregnancy were also evaluated. Women were stratified according to ISSHP criteria [43,44] in preeclamptic (PE) and normal blood pressure in pregnancy (NBPP); and according to European Society of Hypertension (ESH) and European Society of Cardiology (ESC) criteria [45] in hypertensive after pregnancy (HT), and normotensive after pregnancy (NBPPP).

DNA extraction

Whole blood samples were obtained from patients and controls. Genomic DNA was isolated from whole blood samples, using a non-enzymatic method adapted from D.K. Lahiri and J.I. Nurnberger Jr. (1991) method [46].

Genotyping AQP3 (rs2231231) and AQP7 (rs2989924) genes

Real-time PCR reactions were carried out using a LightCycler480 System (Roche), the TaqMan SNP PCR Master Mix (Applied Biosystems), the following specific TaqMan pre-

designed SNP primers: AQP3 (C_2736451_20) and AQP7 (C_11825488_10) (Applied Biosystems) and 35ng of DNA. Genotypes were analyzed and determined by the Roche software AQP3 genotype polymorphisms will be represented by C/C or C/A or A/A; and, AQP7 by T/T or T/C or C/C.

Genotyping the CYBA (p22phox; rs4673) gene

Polymorphic regions were amplified by PCR in a 25- μ l reaction mixture consisting of PCR Nucleotide Mix (Thermo Scientific® DreamTaq Green) containing 10mM dNTPs. 1.5 mM MgCl₂; 1UTaq polymerase; 10 mM of each primer; DMSO and 100 ng of genomic DNA. To amplify the 348-bp polymorphic region of the CYBA gene, we used the following primers: forward primer 5' TGCTTGTGGGTAAACCAAGGCCGGTG 3' and reverse primer 5' AACACTGAGGTAAGTGGGGGTGGCTCCGT 3', followed by digestion with *RsaI* that recognizes the nucleotide sequence GTAC. PCR conditions involved an initial denaturation of DNA at 95 °C for 5 min. followed by 35 cycles of amplification at 94 °C for 45 s (melting), 54 °C for 1 min (annealing), and 72 °C for 30 s, and final extension at 72 °C for 10 min. The resulting product was digested with the restriction endonuclease at 37 °C for 16 h according to the manufacturer's instructions, and digestion products were analyzed after electrophoresis in 3 % agarose gel stained with ethidium bromide (10 μ g/ml). Restriction enzyme digestion results in either 348-bp product (allele C) or a 188- and 160-bp product (allele T). *p22phox* genotype polymorphisms will be represented by C/C or C/T or T/T.

Genotyping the CAT (rs1001179) gene

Polymorphic regions were amplified by polymerase chain reaction (PCR) in a 25- μ l reaction mixture consisting of PCR Nucleotide Mix (Thermo Scientific® DreamTaq Green) containing 10 mM dNTPs, 1.5 mM MgCl₂, 1 U Taq polymerase, 10 mM of each primer; and 100 ng of genomic DNA. To amplify the 185-bp polymorphic region of the CAT gene, we used the following primers: forward primer 5'-AGAGCCTCGCCCCGCCGGACCG-3' and reverse primer 5'-TAAGAGCTGAGAAAGCATAGCT-3', followed by digestion with *SmaI* that recognizes the nucleotide sequence CCCGGG. PCR conditions involved an initial denaturation of DNA at 95 °C for 3 min, followed by 5 cycles of amplification at 94 °C for 1 min (melting), 51 °C for 1 min (annealing), and 72 °C for 1 min, and 35 more cycles of amplification at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s and final extension at 72 °C for 10 min. The resulting product was digested with the restriction endonuclease at 37 °C for 16 h and 65 °C for 20min according to the manufacturer's recommendations, and digestion products were analyzed after electrophoresis in 3 % agarose gel stained with ethidium bromide (10 μ g/ml). Restriction enzyme digestion results in a 185-bp product (allele T) or a 155- and 30-bp product (allele C). The genotypes for this polymorphism will be represented by C/C or C/T or T/T.

Genotyping the NOS3 (VNTR - intron 4) gene

Amplification of DNA samples for polymorphic analysis of the number of tandem repeats (VNTR) in intron 4 (27 bp tandem repeat) was performed by polymerase chain reaction (PCR) in a 25- μ l reaction mixture consisting of PCR Nucleotide Mix (Thermo Scientific® DreamTaq Green) containing 10 mM dNTPs, 1.5 mM MgCl₂, 1 U Taq polymerase, 10 mM of each primer, and 200 ng of genomic DNA. To amplify the polymorphic region of the NOS3 gene we used the following primers: forward primer 5'-AGGCCCTATGGTAGTGCCTT-3' and reverse primer 5'-TCTCTTAGTGCTGTGGTCAC-3'. PCR conditions involved 35 cycles of 94 °C for 30s (denaturation), followed by 53 °C for 30s (annealing), and 72 °C for 45s and final extension at 72 °C for 5 min. The resulting product was analyzed by electrophoresis in 3 % agarose gel stained with ethidium bromide (10 μ g/ml) followed restriction fragment length analysis. Amplification products result in either 420-bp product (allele B) or a 393-bp product (allele A). The genotypes of this polymorphism will be represented by B/B or B/A or A/A.

Genotyping the MPO (rs2333227) gene

Polymorphic regions were amplified by polymerase chain reaction (PCR) in a 25- μ l reaction mixture consisting of PCR Nucleotide Mix (Thermo Scientific® DreamTaq Green) containing 10 mM dNTPs, 1.5 mM MgCl₂, 1 U Taq polymerase, 10 mM of each primer, and 100 ng of genomic DNA. To amplify the polymorphic region of the MPO gene, we used the following primers: forward primer 5'-GTATAGGCACACAATGGTGAG-3' and reverse primer 5'-GCAATGGTTCAAGCGATTCTTC-3'. PCR conditions involved an initial denaturation of DNA at 94 °C for 2 min, followed by 35 cycles of amplification at 94 °C for 1 min (melting), 59 °C for 1 min (annealing) and 72 °C for 1 min (extension). The resulting product was digested with the restriction endonuclease *SsiI* at 37 °C for 16 h according to the manufacturer's recommendations, and digestion products were analyzed following electrophoresis in 3 % agarose gel stained with ethidium bromide (10 μ g/ml) for 90 min with 85 volts. With this process, we are able to differentiate three genotypes: the GG genotype gives rise to three bands (169, 120, 61 bp); the AA genotype appears as two bands, one with 289 bp, and other with 61 bp; the GA genotype shows all four bands (289, 169, 120, 61 bp).

Circulating Cardiovascular Risk Biomarkers Determination

The different circulating biomarkers were determined by enzyme-linked immunosorbent assay (ELISA - R&D Systems Inc.), as myeloperoxidase (MPO, ng mL⁻¹), Nitric oxide metabolites (NOx, μ mol L⁻¹) and nitrites (μ mol L⁻¹), transaminases - AST (Aspartate transaminase, UI L⁻¹), and ALT (Alanine transaminase, UI L⁻¹) were determined by conventional standardized methods. Classic biomarkers as serum lipids and lipoproteins: total cholesterol (t-cholesterol, mg dL⁻¹), non-HDL cholesterol (cholesterol, mg dL⁻¹), and HDL and

LDL cholesterol were measured by using automated enzymatic assays (ABX Diagnostic) and apolipoprotein A and B (Apo A and B, mg dL⁻¹) by using automated immunoturbidimetric assays (ABX Diagnostic). Serum C-reactive protein (CRP, mg L⁻¹) was assessed by an immunoenzymatic method (adaptation of the method of Highton and Hessian, 1984 [47]). Red cell distribution width (RDW%), glucose, triacylglycerols (TAG, mg dL⁻¹) and mean corpuscular hemoglobin concentration (MCHC, g dL⁻¹) were collected from patient hospital records.

Blood Pressure and Anthropometric Parameters Evaluation

Systolic and diastolic blood pressure in mmHg (BP) and pulse were measured by an oscillometric method. Anthropometric parameters such as weight (Kg) and hip (HC, cm) and waist circumference (WC, cm) were evaluated using classic measurement instruments.

Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics version 25. The observed genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium with the chi-square goodness-of-fit test. This test was also used to evaluate the significant differences between the two populations, in order to know if the odds ratio (OR) test was justifiable. OR for HT risk and the corresponding 95% confidence intervals (CI) were calculated using logistic regression analysis adjusted for age. This test was applied to each polymorphism to analyze its risk factor individually; it was also applied to the association of both polymorphisms to analyze the functional possible epistatic relation between the genes of these enzymes and how it may affect the risk for HT. *p* values <0.05 were considered statistically significant.

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Chapter IV

Dietary Modulation of Aquaporins Expression

4. Modulation of aquaporin gene expression by n-3 long-chain PUFA lipid structures in white and brown adipose tissue from hamsters

This chapter is adapted from one publication in a peer review journal:

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[#] Equal contribution

Abstract

EPA (20:5n-3) and DHA (22:6n-3) fatty acids have weight-reducing properties with physiological activity depending on their molecular structure – that is, as TAG or ethyl esters (EE). Aquaporins (AQP) are membrane protein channels recognized as important players in fat metabolism, but their differential expression in white adipose tissue (WAT) and brown adipose tissue (BAT), as well as their modulation by dietary n-3 long-chain PUFA (LCPUFA) such as EPA and DHA, has never been investigated. In this study, the transcriptional profiles of AQP3, AQP5, AQP7 and selected lipid markers of WAT (subcutaneous and visceral) and BAT (interscapular) from hamsters fed diets containing n-3 LCPUFA in different lipid structures such as fish oil (FO, rich in EPA and DHA in the TAG form) and FO-EE (rich in EPA and DHA in the EE form) were used and compared with linseed oil (LSO) as the reference group. A clear effect of fat depot was observed for AQP3 and leptin (LEP), with the lowest values of mRNA found in BAT relative to WAT. The opposite occurred for PPAR α . AQP7 was affected by diet, with FO-fed hamsters having higher mRNA levels compared with LSO-fed hamsters. The relative gene expression of AQP5, adiponectin (ADIPO), GLUT4 and PPAR γ was influenced by both fat tissue and diet. Taken together, our results revealed a differential expression profile of AQP and some markers of lipid metabolism in both WAT and BAT in response to feeding n-3 LCPUFA in two different structural formats: TAG and EE.

Keywords: Aquaporins, Markers of lipid metabolism, n-3 Long-chain PUFA, Brown adipose tissue, White adipose tissue

Introduction

Excessive intake of a Western-style diet rich in SFA and n-6 long-chain PUFA (LCPUFA) and poor in n-3 LCPUFA is one of the major factors promoting the onset of obesity [1,2]. In contrast, EPA (20:5n-3) and DHA (22:6n-3) acids were reported, more than just once, as having weight-reducing properties [3,4,5]. The physiological activity of n-3 LCPUFA may depend on their specific lipid structure. Although not consensual, the TAG form seems to be more bioavailable and, therefore, better absorbed and assimilated than the ethyl esters (EE) form [6]. Adipose tissue plays a central role in

regulating energy balance through its metabolic, cellular and endocrine functions. It has been classified into anabolic white adipose tissue (WAT) and catabolic brown adipose tissue (BAT) [7]. However, certain differentiated adipocytes can undergo a process known by 'browning', switching its metabolic phenotype from typical WAT to those resembling BAT and altering between anabolic and catabolic states [7]. Subcutaneous WAT and visceral WAT are metabolically distinct. Subcutaneous fat expands predominantly by hyperplasia and appears protective through enhanced adipogenic capacity [8]. In opposition, visceral fat expansion occurs mainly by hypertrophy with great infiltration of macrophages [9]. Visceral adipose tissue activity is also regulated by a great number of glucocorticoid receptors β -adrenoceptors and a lower number of insulin receptors [10,11]. Hence, visceral fat accumulation is important in clinical terms because it is more closely associated with the metabolic syndrome. Conversely, BAT constitutes a metabolically active tissue responsible for non-shivering thermogenesis and depletion of excess energy content [12].

At birth, human newborns have a considerable amount of BAT in their body (1–5 % total body weight, meaning a mass of 35–200 g), which is implicated in heat generation, whereas skeletal muscle is not able to make any movements to produce heat [13]. In puberty, this function declines, and is associated with sex-dependent hormones. Recently, BAT was also found to be functionally and metabolically active in adult humans [14], and it is more developed in people exposed to continuous cold, such as outdoor workers [15]. In fact, in adults, BAT can amount to <100 g in average, which might seem few, but when activated it can be able to consume 3–4 kg of WAT in 1 year [14]. Consequently, BAT appears to be preventive against metabolic disorders associated with obesity [16]. In fact, the differential expression of brown adiposity phenotype may, in part, explain why some individuals are more prone to obesity than others. It actually underlies the new concept of metabolically healthy obesity. Aquaporins (AQP) are a family of transmembrane protein channels that facilitate the permeation of water and small solutes, such as glycerol, across cell membranes [17,18]. According to their primary sequence and permeability characteristics, AQP are divided into three sub-groups: (i) orthodox or classical AQP (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8), primarily water selective [17,18] (ii) aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10), permeable to small uncharged solutes, such as glycerol and urea in addition to water [17,18,19]; (iii) unorthodox or S-AQP (AQP11 and AQP12), found in intracellular membranes, with lower sequence similarity to the

other mammalian AQP and suggested as permeable to water (AQP11 and AQP12) and glycerol (AQP12) [20,21]. In the adipose tissue, some AQP isoforms may have a pivotal role in controlling fat accumulation [22,23] and have been implicated in obesity and related metabolic complications [24]. Among the various mammalian aquaglyceroporins, AQP7 is the most representative glycerol channel and was the first to be detected in human and mouse adipose tissue [25,26]. Impaired glycerol transport through AQP7 (an aquaglyceroporin) leads to glycerol retention within adipose tissue, ultimately leading to acceleration of TAG synthesis and accumulation, which has been correlated to obesity development [25,27,28]. However, the presence of other aquaglyceroporins in addition to AQP7 has been more recently reported [23,29]. AQP3 was identified in both subcutaneous and visceral human fat depots but with a stronger expression in the stromal vascular tissue adjacent to adipose [23,30,31]. Interestingly, AQP3 was the isoform with higher expression in human visceral adipose tissue followed by AQP9 and AQP7, with AQP3 gene expression being found in both fully mature adipocytes and stroma vascular fraction (SVF) cells. AQP3 is highly expressed in preadipocytes, macrophages and other cells within the SVF (leucocytes, neutrophils, erythrocytes and fibroblasts). Similar to AQP7, AQP3 is up-regulated by catecholamines, suggesting that, together with other aquaglyceroporins, it may be relevant to regulate paracellular transport of glycerol between the bloodstream and the interstitium of WAT [23,24].

More recently, our group detected the expression of AQP5 in 3T3-L1 adipocytes and showed that besides a role in water transport AQP5 depletion reduces lipid droplets content and impairs adipocyte differentiation [30]. However, AQP5 involvement in the obesity mechanisms and the differential expression of these AQP isoforms in WAT (subcutaneous and visceral) and BAT remains unclear. In this work, we investigated the variability of AQP (AQP3, AQP5, AQP7) gene expression across subcutaneous WAT, visceral WAT and interscapular BAT, which might be associated with adipose tissue depot's own location and their different metabolic functions. In addition, to further investigate whether the body fat reduction properties of n-3 LCPUFA *in vivo* are correlated with AQP expression levels in the adipose tissue, we compared the AQPs' transcriptional profile of WAT (subcutaneous and visceral) and BAT from hamsters fed diets containing specific n-3 LCPUFA molecular structures: fish oil (FO, rich in EPA and DHA in the TAG form) and FO-EE (rich in EPA and DHA in the EE form), v. linseed oil (LSO, rich in α -linolenic acid (ALA), 18 : 3n-3) used as control. The

expression of some markers of lipid metabolism, namely adiponectin (ADIPO), leptin (LEP), GLUT4, PPAR α and PPAR γ , was also characterized in the different adipose tissue samples.

Results

Effect of fat depot on gene expression levels of aquaporins and markers of lipid metabolism

In quantitative terms and comparing the transcriptional profile of AQP3, AQP5 and AQP7 in hamster's fat tissues, the levels of AQP3 mRNA were predominant over AQP7 and AQP5 (AQP3>AQP7>AQP5) in all fat depots. A clear effect of fat depot was observed for AQP3 and some markers of lipid metabolism, such as LEP and PPAR α , meaning that, regardless of the dietary treatment, the transcriptional profile of this gene was divergent among adipose tissues. This effect of fat depot is most certainly associated with the lower transcriptional profile of AQP3 in BAT relative to subcutaneous ($P<0.001$) and visceral WAT ($P<0.001$) (Figure 4.1). Identical variations were found for LEP in BAT relative to subcutaneous ($P<0.001$) and visceral ($P<0.001$) WAT, and also between WAT ($P=0.007$) (Figure 4.2). Conversely, PPAR α is highly expressed in BAT in comparison with residual levels of mRNA expression found in both subcutaneous ($P<0.001$) and visceral ($P<0.001$) WAT (Figure 4.3). The variation pattern between subcutaneous and visceral WAT is similar for some genes, including AQP3, AQP5, AQP7 and LEP (Figure 4.1 and 2, $P>0.05$).

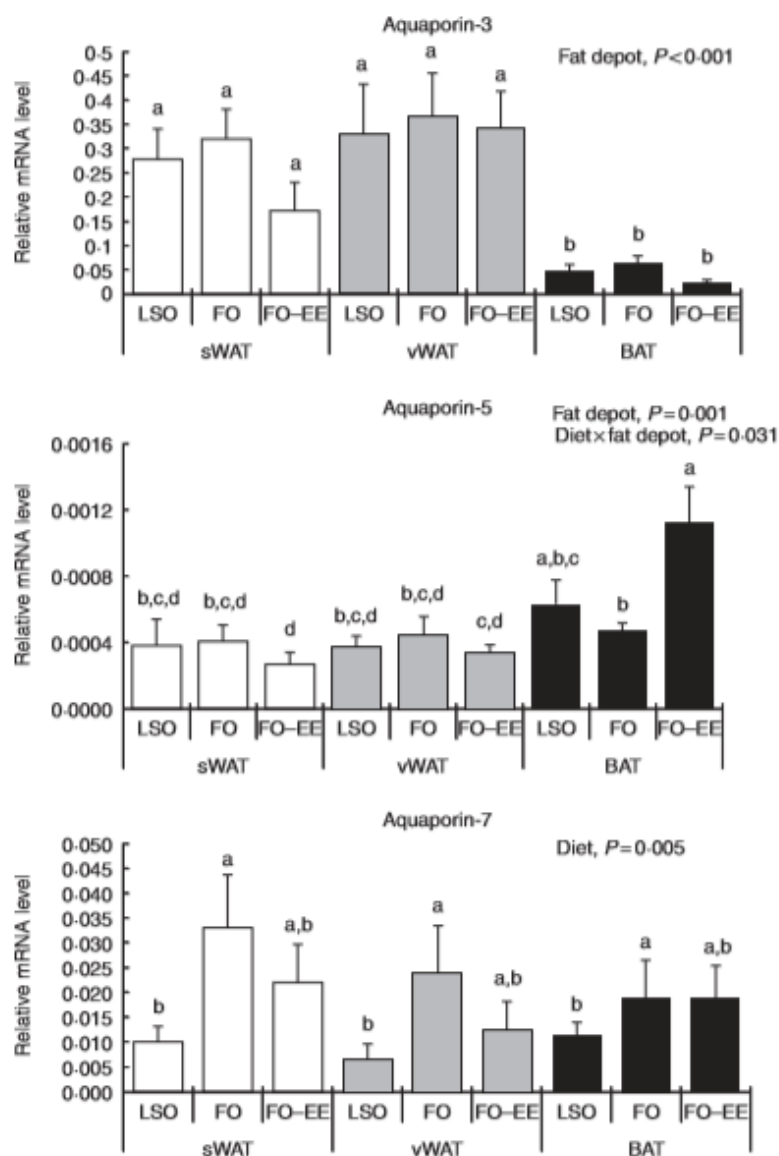


Figure 4.1. Effect of fat depot, diet and diet $\times$ fat depot interaction on the relative expression levels of aquaporin-3, aquaporin-5 and aquaporin-7 in the subcutaneous white adipose tissue (sWAT, white bars), visceral white adipose tissue (vWAT, gray bars) and brown adipose tissue (BAT, black bars) from hamsters fed linseed oil (LSO), fish oil (FO) and FO-ethyl esters (EE). Values are means, with their standard errors represented by vertical bars. a,b,c,d Mean values with unlike letters were significantly different (Tukey's post hoc, $P < 0.05$).

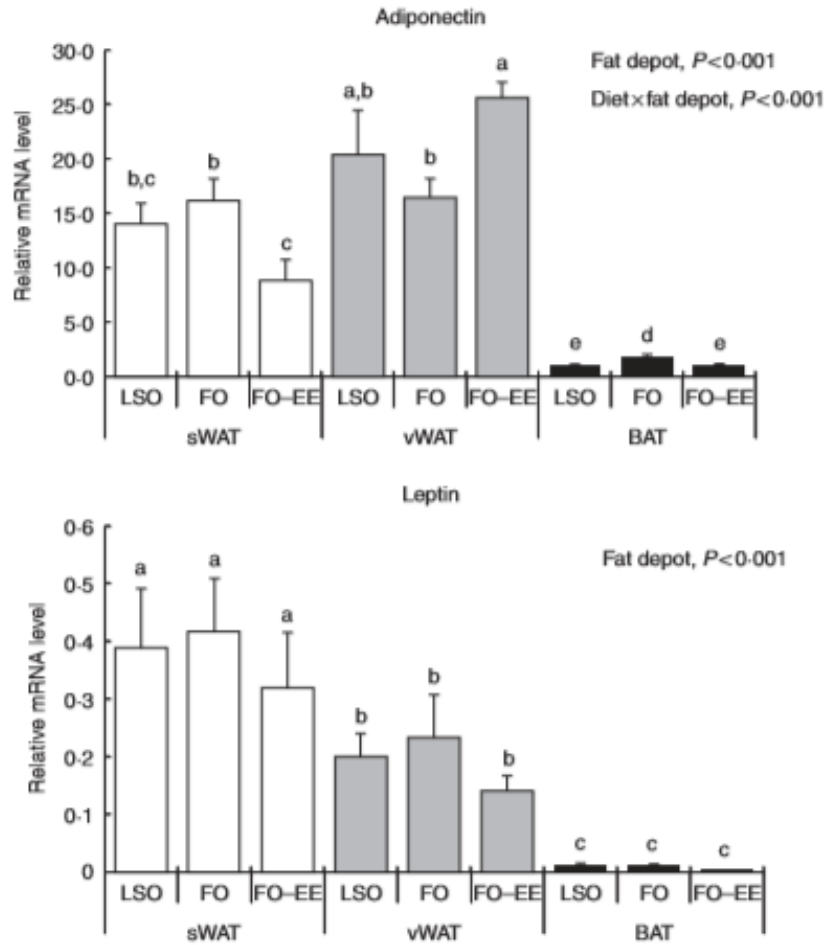


Figure 4.2. Effect of fat depot, diet and diet $\times$ fat depot interaction on the relative expression levels of adiponectin and leptin in the subcutaneous white adipose tissue (sWAT, white bars), visceral white adipose tissue (vWAT, gray bars) and brown adipose tissue (BAT, black bars) from hamsters fed linseed oil (LSO), fish oil (FO) and FO–ethyl esters (EE). Values are means, with their standard errors represented by vertical bars. a,b,c,d,e Mean values with unlike letters were significantly different (Tukey’s post hoc, $P < 0.05$).

Effect of diet on gene expression levels of aquaporins and markers of lipid metabolism

The only gene affected solely by diet was AQP7. In fact, increased mRNA levels of this AQP were found in hamsters fed FO relative to LSO ($P = 0.008$) but not in hamsters fed FO–EE relative to LSO ($P = 0.050$), suggesting a positive effect of EPA and DHA when combined as TAG in AQP7 gene expression (Figure 4.1). No changes were detected for AQP7 gene expression between FO and FO–EE-fed hamsters (Figure 4.1, $P = 0.249$).

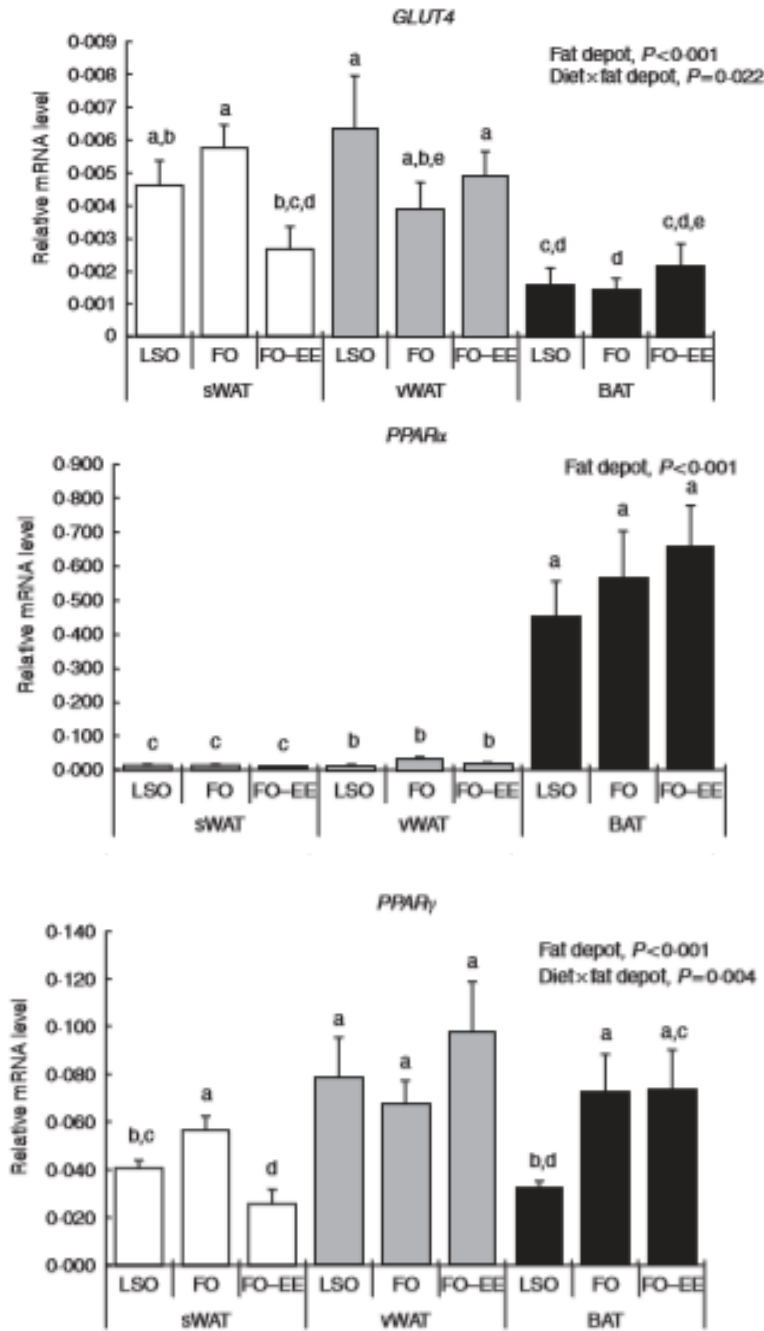


Figure 4.3. Effect of fat depot, diet and diet×fat depot interaction on the relative expression levels of GLUT4, PPAR α and PPAR γ in the subcutaneous white adipose tissue (sWAT, white bars), visceral white adipose tissue (vWAT, gray bars) and brown adipose tissue (BAT, black bars) from hamsters fed linseed oil (LSO), fish oil (FO) and FO–ethyl esters (EE). Values are means, with their standard errors represented by vertical bars. a,b,c,d,e Mean values with unlike letters were significantly different (Tukey's post hoc, $P < 0.05$).

Interaction effect of fat depot×diet on gene expression levels of aquaporins and markers of lipid metabolism

The genes for AQP5, ADIPO, GLUT4 and PPAR γ were affected by the interaction between diet and fat, as illustrated in Figure 4.1–4.3. An interaction effect between diet and fat was found for the expression level of AQP5 (Figure 4.1, $P=0.031$). The reason for this is that BAT presented increased mRNA levels of AQP5 than subcutaneous and visceral WAT when hamsters were fed an FO–EE diet. For ADIPO, higher mRNA levels were observed in the FO diet relative to FO–EE in subcutaneous WAT and BAT, whereas the inverse was found for visceral WAT (Figure 4.2, $P<0.001$). GLUT4 was also affected by an interaction between diet and fat (Figure 4.3, $P=0.022$), which is explained by decreased mRNA levels in the FO–EE diet in subcutaneous WAT, not verified in BAT and visceral WAT. For PPAR γ , hamsters fed FO–EE had lower mRNA levels than FO in subcutaneous WAT but identical in visceral WAT and BAT, justifying the interaction detected between diet and fat (Figure 4.3, $P=0.004$).

Pearson's correlation coefficients among genes per fat depot

Table 4.1 presents the Pearson's correlation coefficients (r) for gene expression levels in subcutaneous WAT, visceral WAT and BAT. It is noteworthy that all positive correlations found were moderate ($0.7 \geq r \geq 0.3$). In subcutaneous WAT, AQP3 was positively correlated with ADIPO ($P<0.01$) and AQP7 ($P<0.01$). ADIPO correlated positively with PPAR γ ($P<0.01$), PPAR α ($P<0.01$) and LEP ($P<0.01$). A positive correlation was observed between GLUT4 and PPAR γ ($P<0.001$), GLUT4 and PPAR α ($P<0.05$) and between PPAR α and PPAR γ ($P<0.01$) (Table 4.1). In visceral WAT, fewer correlations were found: AQP3 was positively correlated with AQP7 ($P<0.05$) and also with AQP5 ($P<0.05$) (Table 4.1). In BAT, AQP7 correlated positively with PPAR γ ($P<0.05$) and LEP ($P<0.05$). ADIPO was correlated with both PPAR γ ($P<0.01$) and LEP ($P<0.05$). PPAR α correlated with both GLUT4 ($P<0.05$) and PPAR γ ($P<0.01$) (Table 4.1).

Table 4.1. Pearson's correlations coefficients among gene expression levels in subcutaneous white adipose tissue, visceral white adipose tissue and brown adipose tissue

	<i>PPARγ</i>	<i>PPARα</i>	<i>GLUT4</i>	<i>LEP</i>	<i>ADIPO</i>	<i>AQP7</i>	<i>AQP5</i>
Subcutaneous white adipose tissue							
<i>AQP3</i>	0.329	0.053	-0.108	0.087	0.570**	0.660**	0.310
<i>AQP5</i>	0.036	-0.182	-0.173	-0.018	0.045	0.122	
<i>AQP7</i>	0.335	0.059	0.084	-0.048	0.340		
<i>ADIPO</i>	0.630**	0.573**	0.311	0.562**			
<i>LEP</i>	0.269	0.036	0.035				
<i>GLUT4</i>	0.672***	0.489*					
<i>PPARα</i>	0.574**						
<i>PPARγ</i>							
Visceral white adipose tissue							
<i>AQP3</i>	0.072	-0.028	-0.198	-0.166	0.018	0.500*	0.438*
<i>AQP5</i>	0.097	0.351	-0.014	0.149	0.199	0.077	
<i>AQP7</i>	0.361	-0.044	-0.089	0.380	-0.024		
<i>ADIPO</i>	0.240	-0.218	-0.197	0.223			
<i>LEP</i>	0.119	-0.078	0.015				
<i>GLUT4</i>	-0.063	-0.053					
<i>PPARα</i>	-0.049						
<i>PPARγ</i>							
Brown adipose tissue							
<i>AQP3</i>	0.012	-0.182	-0.420	-0.194	0.192	0.332	-0.133
<i>AQP5</i>	-0.151	0.057	0.095	-0.327	-0.386	-0.048	
<i>AQP7</i>	0.503*	0.796	0.667	0.137*	0.069		
<i>ADIPO</i>	0.574**	0.302	0.015	0.530*			
<i>LEP</i>	0.129	0.244	0.280				
<i>GLUT4</i>	-0.069	0.500*					
<i>PPARα</i>	0.594**						
<i>PPARγ</i>							

LEP, leptin; *ADIPO*, adiponectin; *AQP*, aquaporins.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

AQP are membrane protein channels recognized as important players in controlling fluid and fat homeostasis. Although AQP7 has been reported to be involved in obesity, the role of other AQP in fat-related metabolic disorders is still unclear. Moreover, their differential expression in WAT and BAT, as well as their modulation by dietary n-3 LCPUFA, such as EPA and DHA in distinct molecular structures, has never been investigated. In this experimental trial, hamsters' final body weight and weight gain did not vary among dietary groups [6], as well as adipose tissue weight from subcutaneous WAT, visceral WAT and interscapular BAT [32], as all diets contained the same amount of fat. These data agree with similar values of hamsters' daily feed intake. The abundance of n-3 LCPUFA in human fat does not increase notably even after supplementation [33]. EPA and DHA are not particularly stored long-term in TAG; rather, they may be partitioned to oxidation pathways or storage in other lipid fractions,

such as plasma phospholipids, cell phospholipids and erythrocytes, all of them having a notably higher abundance of both EPA and DHA than adipose tissue [5,6,32].

AQP5 is a classical water channel, whereas AQP3 is an aquaglyceroporin that facilitates permeation of glycerol in addition to water [34]. As expected, we found higher levels of AQP3 expression in WAT in relation to BAT, possibly owing to the main role of WAT in energy storage in the form of TAG. In BAT, AQP3 expression was slightly decreased, although not reaching statistical significance, when hamsters were fed the FO–EE diet, which probably means that the EE molecular form promotes less glycerol efflux in brown adipocytes in comparison with TAG. Interestingly, when comparing the transcriptional profile of both AQP, an inverse pattern of variation was found for AQP3 and AQP5 between FO and FO–EE diets in BAT. Although AQP5 does not contribute to glycerol fluxes, it was shown to be important for adipocyte differentiation [30], and thus a complementary role of these two isoforms for intracellular TAG accumulation cannot be ruled out.

Among the various mammalian aquaglyceroporins, AQP7 is the most representative glycerol channel and was the first to be detected in the adipose tissue from human and rodents [35,36], although predominance of AQP3 in human visceral adipose tissue has been reported [37]. In the adipose tissue from hamsters the higher expression was found for AQP3, rather than for AQP7. In the overall picture, AQP7 expression was only affected by diet, with higher levels of mRNA found in the FO dietary treatment in comparison with LSO and FO–EE, suggesting that EPA and DHA when combined as TAG activate the efflux of glycerol from the adipocytes via AQP7, possibly leading to a positive decrease in lipid droplets size. Curiously, the expression of AQP7 was kept unchanged in all three adipose tissues (there was no fat depot effect), confirming its conservative role in glycerol release from adipocytes that ensure lipid homeostasis [35].

ADIPO has emerged as the most abundant adipocyte product, thereby redefining adipose tissue as a key component of the endocrine system [38]. In obesity, the circulating levels of ADIPO fall while LEP levels rise, suggesting that the regulation of these two adipocyte-derived hormones may be simultaneously influenced by common obesity-related factors [39]. In fact, ADIPO and LEP are adipokines responsible for normal adipocyte phenotype and function [40]. The regulation pattern of gene expression of ADIPO in subcutaneous and visceral WAT was similar, perhaps subjective to common control of energy balance. The same does not apply for LEP. LEP

administration has been shown to down-regulate AQP7 expression in murine subcutaneous adipocytes [41]. We found no correlation (positive or negative) between LEP and AQP7 mRNA levels in subcutaneous WAT, but instead a positive correlation in BAT that deserves further investigation. In BAT, both ADIPO and LEP showed lower expression, indicating that ADIPO and LEP have in WAT their major contributor. In fact, WAT is known to be the most relevant tissue that accounts for endocrine factors, such as ADIPO and LEP in the bloodstream [42]. However, ADIPO production by BAT, although in lower amounts, is well documented [43,44]. It is now evident that the LEP synthesis also takes place in BAT, although the physiological role of LEP produced by brown fat is still unclear.

Conversely, PPAR α was highly expressed in BAT, which certainly relates to fatty acid β -oxidation [45] occurring in brown adipocytes as BAT is primarily a thermogenic organ. In turn, PPAR γ stimulates glycerol transport and glycerol phosphorylation [46,47]. Moreover, AQP7 is a direct PPAR γ target gene in adipocytes [46,48]. This evidence is not supported by the moderate positive correlation found between AQP7 and PPAR γ mRNA levels in BAT, rather than what would be expected to be found in WAT. Furthermore, we have demonstrated that, in subcutaneous WAT, PPAR α and PPAR γ expression correlated significantly with the expression of GLUT4, which is an essential gene in the lipogenesis pathway [40]. These findings demonstrate a relationship between PPAR expression and the expression of other genes of lipid metabolism and support the concept that PPAR α and PPAR γ activators may regulate fatty acid metabolism in the adipose tissue.

Variations of response across fat depots (subcutaneous WAT, visceral WAT and BAT) suggest that the features of adipose tissue responsible for adipocyte cells and differentiation may not be homogeneous, both in nature and distribution [49]. Visceral fat accumulation, as supported by PPAR γ up-regulation, which is the key regulator of adipocytes differentiation, is important in clinical terms because it is more closely linked to the metabolic syndrome owing to its inflammatory features. Conversely, brown adipocytes constitute a metabolically active tissue responsible for non-shivering thermogenesis, depletion of excess energy content being less inflammatory than WAT. These features are in line with PPAR α , ADIPO and LEP gene variations. BAT is associated with metabolic health owing to its oxidative capacity and appears to be effective in the protection against metabolic disorders associated with obesity and diabetes. In addition, the structure of combined EPA and DHA as EE reduced the gene

expression of ADIPO, GLUT4 and PPAR γ in subcutaneous WAT, as well as ADIPO in BAT. The only exception was ADIPO, whose mRNA levels were found to be increased in the FO–EE diet relative to FO in visceral WAT. On the basis of the current research, the physiological activity of n-3 LCPUFA and, in particular, the potential effect of a specific fatty acid depends both on its structure and administration form. There seems to be a difference in the apparent bioavailability of n-3 LCPUFA, such that the TAG form is more bioavailable than the EE form [50,51,52]. This interpretation is in line with our own findings. TAG define three fatty acid esterified (bonded) to a glycerol backbone and is the natural molecular form that makes up virtually all fats and oils in both animal and plants species, devoid of toxicological effects and more chemically stable than EE [53,54]. The EE form lacks the glycerol backbone needed to reassemble the TAG structure. We speculate that this could be the major obstacle to the efficient absorption of EE form EPA and DHA, impairing in due course the transcriptional profile of these genes.

Conclusions

In this study, we reported differential AQP3, AQP5 and AQP7 gene expression across subcutaneous WAT, visceral WAT and interscapular BAT, which may reflect adipose tissue depot's own location and metabolic function. We also found different patterns of AQP and markers of lipid metabolism expression between WAT (subcutaneous and visceral) and BAT when hamsters were fed specific n-3 LCPUFA molecular structures: FO (rich in EPA and DHA in the TAG form) and FO–EE (rich in EPA and DHA in the EE form). Among the AQP isoforms, AQP7 stands out as the more promising target for developing new anti-obesity drugs owing to its conservative role across WAT and BAT.

Materials and Methods

Ethics statement

The experimental procedures were reviewed by the Ethics Commission of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority (Direcção Geral

de Alimentação e Veterinária, Portugal), following the appropriate European Union guidelines (2010/63/EU Directive).

Animals and diets

Besides having BAT in considerable amounts owing to hibernation, the lipid metabolism of hamsters is more similar to humans than mice and rats [55,56], thus making it possible to extrapolate results to humans. In all, 10-week-old Golden Syrian male hamsters were purchased from Charles River (Charles River Laboratories). Hamsters were housed one per cage under a 14/10-h light/dark cycle schedule, synchronised with natural daylight, with controlled temperature of 20–24°C in a certified facility at the Faculty of Veterinary, University of Lisbon. During the first week, all animals were fed a standard diet to minimise stress and stabilise all metabolic conditions. After this period, twenty-four hamsters were assigned to three body-weight-matched groups, with eight animals each: the LSO group, rich in ALA, without EPA or DHA, taken as the control group; the FO group, a commercially available oil rich in TAG with EPA and DHA; and the FO–EE group. The final sum of EPA and DHA was identical across FO and FO–EE dietary treatments [6,32]. Diets were manufactured by the Experimental Diets Unit from the University of Almería in Spain. The detailed composition of the experimental diets, in terms of proximate chemical composition and fatty acids, has been published elsewhere [6]. Briefly, all diets were based on AIN-93M formulation with modified lipid fractions. Each diet contained the following (g/100 g feed): casein (14·0), maize starch (46·6), maltodextrin (15·5), sucrose (10·0), cellulose (5·0), soyabean oil (4·0), L-cystine (0·18), AIN-93 mineral mix (3·5), AIN-93M vitamin mix (1·0), choline bitartrate (0·25) and tert-butylhydroquinone (0·0008). Throughout the trial, hamsters had free access to water and food. Body weight and feed intake were recorded twice a week. After 12 weeks of feeding trial, hamsters were fasted for 12 h, weighed before and after the fasting period and euthanised by a mechanical–physical method. Hamsters were placed in a chamber and anaesthetised using a mixture of 20 % of isoflurane in propylene glycol (v/v) for 30 s [57], followed by decapitation with a small animal guillotine, in certified ethical conditions to minimise animal suffering. Subcutaneous and visceral WAT from hamsters' pelvic and retroperitoneal anatomical regions, respectively, as well as BAT from hamsters' interscapular region were excised, weighed and stored at –80°C for subsequent gene expression determination.

Adipocyte RNA extraction and complementary DNA synthesis

Total RNA was isolated and purified from subcutaneous WAT, visceral WAT and BAT using a Qiagen RNeasy lipid tissue mini kit (Qiagen) and stored at –80°C. To exclude possible

DNA contamination, on-column DNA digestion with the RNase-free DNase set (Qiagen) was performed. All procedures were based on the manufacturer's protocol. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop1 ND-2000c (ThermoFisher Scientific). The ratios 260/280 and 260/230 nm were also determined to assess the purity of RNA samples and the presence of contaminants. Only samples that fulfill the established quality parameters (260/280 and 260/230 nm ratios were used as purity measurements for protein and solvent presence, respectively, considering ratios between 1.8 and 2.2) proceeded to complementary DNA (cDNA) synthesis step. To generate cDNA for quantitative PCR, 750 ng of total RNA was reverse-transcribed for 2 h at 37°C using the first strand cDNA synthesis kit (NZYtech). Specific primers for real-time quantitative PCR were designed for seven genes (AQP3, AQP5, AQP7, ADIPO, GLUT4, PPAR α and PPAR γ). The DNA sequences of the golden hamster (*Mesocricetus auratus*) genes were obtained from GenBank and then submitted to the Primer3 software (<http://primer3.ut.ee/>) to generate primers. Primer3 parameters were set in order to obtain the best pair of primers in size (20–27 bp), melting temperature (50–65°C), % GC (50–60 %) and product size range (75–200 bp). The characterisation of the selected genes used in real-time quantitative PCR is described in Table 4.2.

Table 4.2. Gene-specific primer sequences used for real-time-quantitative PCR.

Gene symbol	Full gene name	GenBank accession no.	Forward/reverse primers	Product size (bp)
<i>AQP3</i>	Aquaporin-3	XM_005078855.2	F: 5'-CCAACAATGAGCTTATCGTCTCCG-3' R: 5'-CAGAACACACACGATGAGGGAG-3'	137
<i>AQP5</i>	Aquaporin-5	XM_005067227.2	F: 5'-GGTGGTCATGGATCGGTTTCA-3' R: 5'-GAAGAGCAGGTAGAAAGTAGAGCAG-3'	100
<i>AQP7</i>	Aquaporin-7	XM_013119574.1	F: 5'-GCAGAGGGAGATGGTACGAGAG-3' R: 5'-GTCTCCTAGAACCATATGAGCCAC-3'	100
<i>ADIPO</i>	Adiponectin	GQ355976.1	F: 5'-CTCTTCACCTTCGACCAGTATCAG-3' R: 5'-CTGCATAGAGTCCACTGTAATCCC-3'	139
<i>LEP</i>	Leptin	XM_005078071.2	F: 5'-CCAAAACCCCTCATCAAGACCA-3' R: 5'-AGCCCAGGAATGAAGTCCAA-3'	106
<i>GLUT4</i>	Solute carrier family 2 (facilitated GLUT), member 4	XM_005067520.2	F: 5'-ATGGCTGTCGCTGGTTTCTC-3' R: 5'-AAGCAGGAGGACGGCAAATA-3'	117
<i>PPARα</i>	PPAR α	NM_001281543.1	F: 5'-TGAGGAAGCCGTTCTGTGAC-3' R: 5'-GGTGTCATCTGGATGGTTGC-3'	221
<i>PPARγ</i>	PPAR γ	XM_013110341.1	F: 5'-GAGGGCGATCTTGACAGGAA-3' R: 5'-GATGGCCACCTCTTTGCTCT-3'	139
Housekeeping genes				
<i>ACTB</i>	β -Actin	NM_001281595.1	F: 5'-GCCAACCCTGAAAAGATGACC-3' R: 5'-GTACGACCAGAGGCATACAGG-3'	104
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	DQ403055.1	F: 5'-CAGTATGACTCTACCCATGGCAAG-3' R: 5'-CAGTAGACTCCACAACATACTCGG-3'	157
<i>RPL27</i>	Ribosomal protein L27	XM_005070132.2	F: 5'-CATGGGCAAGAAGAAATCGCC-3' R: 5'-GTTTCAGGGCTGGGTCTCTAAAG-3'	155

Real-time quantitative PCR

Real-time quantitative PCR was performed using PowerUp™ SYBR® Green Master Mix (Life Technologies) and Xpert Fast SYBR (GRiSP) to amplify AQP (AQP3, 5 and 7) and markers of lipid metabolism (ADIPO, LEP, GLUT4, PPAR α and PPAR γ), respectively. The

final reaction volume of 20 µl was prepared using 10 µl of SYBR master mix, 3 µl of template cDNA, 2 µl of forward and reverse primers and 3 µl of molecular-biologygrade water. The reaction was performed on a CFX96™ Real-Time PCR Detection System C1000 (BioRad) consisting of an initial denaturation step at 95°C for 3 min, forty-five cycles of denaturation at 95°C for 10 s and annealing/extension at 59 or 62°C (for AQP and markers of lipid metabolism, respectively) for 30 s. The relative expression levels were normalised to reference genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) for AQP3, AQP5 and AQP7; ribosomal protein L27 (RPL27) for ADIPO, LEP, GLUT4, PPARα and PPARγ) and calculated using a variation of the Livak method [58], corrected for variation in amplification efficiency, as described by Fleige & Pfaffl [59].

Statistical analysis

Statistical analyses were carried out with the Statistical Analysis System software, version 9.4 (SAS Institute). All data were presented as means with their standard errors. Data were checked for normal distribution by Kolmogorov–Smirnov test and variance homogeneity using Proc MIXED with a model including the fat depot and diet as fixed effects and the repeated statement considering the group option to accommodate the variance heterogeneity. This analysis was followed by Tukey's multiple comparisons test. Pearson's correlation coefficients were calculated with the Proc CORR procedure to establish linear relationships among gene expression. $P < 0.05$ was considered to be statistically significant.

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Chapter V

Overall Conclusions and Final Remarks

The MetS is an important health concern because of its prevalence, potential severity and consequent costs. It is estimated that over 20 per cent of the adult population worldwide has developed MetS, a syndrome represented by a cluster of common pathologies such as obesity, T2D, IR, dyslipidaemia and fatty liver. The MetS clinical draw is associated with higher risk to develop cardiovascular diseases, as hypertension, atherosclerosis, heart attack and stroke, nowadays the number one cause of death. This syndrome has been revealed challenging because of the difficulty of defining a pathogenesis pattern, its clinical heterogeneity and complexity, and the lack of reliable prognosis biomarkers and therapeutic options. The individual specific phenotype will probably be dependent of the balance between organ specific pathologic effector mechanisms and adaptive allostatic responses ultimately leading to a global homeostatic failure. Clinical and academic facts suggest that failure in β -cells causing hyperinsulinaemia, hepatic defects leading to fatty liver and insulin resistance, and also defects in hypothalamus causing impaired peripheral energy homeostasis, may all prompt the development of MetS. However, although relevant experimental data support the enrolment of these organs in the onset and/or development of MetS, the relevance of each of these entities for prognostic, diagnostic and therapeutic implications needs further clarification. Therefore, identification of potential players in early stages of the syndrome and the organ/tissue in which the alteration occurs is of utmost importance.

This project rose with the premise that aquaglyceroporins, as glycerol channels, are essential for glycerol metabolism by facilitating glycerol release from the adipose tissue and its distribution to various tissues and organs, unveiling these membrane channels as important players in lipid balance and energy homeostasis and highlighting their involvement in a variety of pathophysiological mechanisms including obesity, insulin resistance and diabetes. In order to investigate the involvement of aquaporins in the development of metabolic-related pathologies, different experimental approaches were used and are presented in this thesis.

The first part of results of this thesis (Chapter II) is divided in three subchapters focused on the investigation of the involvement of AQP_s in cell physiology and in the development of pathologies in three distinct cell types: endothelial cells (Subchapter 2.1), pancreatic β -cells (Subchapter 2.2) and macrophages (Subchapter 2.3).

In Subchapter 2.1, knowing that the water channel AQP1 was reported to contribute to endothelial homeostasis and cardiovascular health, we aimed at identifying which aquaglyceroporins are expressed in endothelia and their potential implication in cardiovascular disease. We confirmed the involvement of impaired AQP1-mediated water transport in endothelial dysfunction and activation and identified for the first time AQP3 as the most representative aquaglyceroporin in endothelia. Moreover, we showed that increased SAH in human endothelial downregulates AQP1 decreasing water permeability, but does not affect AQP3 and glycerol permeability. Furthermore, we show that TNF α has a negative effect in AQP1 expression in endothelial cells, suggesting that SAH-induced inflammatory activation might be responsible for the observed decrease of AQP1. Altogether, our results suggest that AQP1 is a candidate player in the setting of endothelial dysfunction and that AQP3, due to high expression level, is the main contributor for metabolic homeostasis.

The Subchapter 2.2 was devoted to the study of the involvement of AQP7 and AQP12 in obesity-induced inflammation, assuming that AQP7 plays a role in insulin exocytosis, and AQP12, possibly involved in inflammation in pancreatic β -cells, here induced by TNF α and LPS. For that purpose, we used a loss-of-function/gain-of-function strategy for each AQP isoform. Our results revealed that, while TNF α downregulates AQP7, LPS induces its upregulation. On the other hand, both TNF α and LPS upregulate AQP12 as well as proinflammatory markers. We demonstrated that AQP7 is closely related to insulin secretion, due to its role as a glycerol channel, and that both overexpression and silencing affects insulin secretion, although overexpression has more severe effects in cell physiology. Moreover, AQP12 is unequivocally entangled with the inflammatory status of the pancreatic β -cell. AQP12 downregulation led to an augmented proinflammatory state while its overexpression significantly improved cell function. Furthermore, we revealed that both isoforms are involved in pancreatic β -cells proliferation, adhesion and migration. AQP7 and AQP12 may be promising targets for new anti-diabetic drug development.

In Subchapter 2.3 we investigated AQPs contribution for the inflammasome priming and activation in macrophages, knowing that inflammasomes are dependent on cell regulatory volume mechanisms and cell volume regulation is generally dependent on AQPs expression. We confirmed the upregulation of AQP9 and AQP3 upon LPS-priming in HPBM and THP-1 cells, respectively, suggesting a link between AQP

dysregulation and disease. In order to studying AQP3 role in inflammation in macrophages, we used an AQP3 inhibitor (Auphen), to block its function. We showed that AQP3 is involved in NF- κ B activation mechanism through TLRs, and that IL-6 release is impaired when AQP3 is blocked. Moreover, we revealed that when AQP3 is functionally inhibited, a reduction in IL-1 β release occurs, indicating that AQP3 is involved in NLRP3-inflamassome. The activation of the NLRP3 inflammasome has been implicated in a variety of metabolic diseases including obesity, atherosclerosis and T2D, suggesting a strong link between inflammation and metabolism. Overall, these new facts underlying the involvement of AQPs in the regulation of the inflammatory response open new scenarios to tailor the modulation of specific AQPs for new pharmacological therapies.

In the second part of this thesis (Chapter III), hypothesizing that AQP3, AQP7 and key oxidative stress-related genes (NOS3, CYBA, MPO and CAT) polymorphisms might be associated to the development of cardiovascular disorders in Caucasian women, we disclosed that the genotypes of NOS3 may modulate the risk of susceptibility to develop preeclampsia during pregnancy while the genotypes of AQP3 and CYBA influence the risk of susceptibility to develop hypertension after pregnancy. In addition, the alleles of risk were associated with abnormal values of several anthropometric, hematologic and biochemical markers of cardiovascular disease. Furthermore, AQP3 and CYBA showed differential genotype distribution among the 4 reclassified groups: PE>HT, PE>NBPPP, NBPP>HT and NBPP>NBPPP. Further studies, expanding these findings to a larger number of patients and combining genetic data with biochemical and anthropometric biomarkers, may be applicable in the prevention of cardiovascular disease.

In the third part of this thesis (Chapter IV), having as a starting point the knowledge that omega-3 fatty acids (n-3 LCPUFA) EPA and DHA have weight-reducing properties and that their biological activity may depend on their molecular structure, i.e. triacylglycerols (TAG) or ethyl esters (EE), we investigated the transcriptional profiles of AQP3, AQP5, AQP7 as well as selected lipid markers of WAT (subcutaneous and visceral) and BAT (interscapular) in hamsters fed diets enriched in EPA/DHA in the TAG form and EPA/DHA in the EE form. First, we found a differential AQP3, AQP5 and AQP7 gene expression in WAT and BAT tissues, which may reflect adipose tissue depot's own location and metabolic function. In addition, we also found different

expression patterns of AQPs and markers of lipid metabolism in different tissues when hamsters were fed specific n-3 LCPUFA molecular structures. Overall, AQP7 stands out as the most promising target for developing new anti-obesity drugs owing to its conservative role across WAT and BAT.

Although some questions remained to be addressed and new ones emerged, at the end of the work here described and discussed, we illustrated the importance of aquaglyceroporins in cell physiology and development of pathologies in several metabolism-related cell types, such as endothelial cells, pancreatic β -cells and macrophages. Firstly, we unveiled the expression of aquaglyceroporins in endothelial cells, linking the glycerol flow between different organs; we gave new evidences on the commitment of AQP7 to insulin secretion and demonstrated a link between AQP12 expression and inflammation in pancreatic β -cells; in addition, we confirmed an involvement of AQP3 in inflammasome priming and activation in macrophages. Secondly, along with oxidative stress-related genes (NOS3 and CYBA), we demonstrated that AQP3 genetic polymorphisms may modulate the susceptibility to develop cardiovascular disease. Finally, we showed that the expression profile of AQPs and some markers of lipid metabolism may be differentially modulated in response to feeding n-3 LCPUFA in two different structural formats (TAG and EE) in both WAT and BAT.

Overall, this work provides new insights on the importance AQPs function and regulation in energy balance and in the development of metabolic-related pathologies. From our results we can conclude that AQPs have great potential as therapeutic targets, although the mechanisms behind the development of metabolic diseases deserve further investigation. Furthermore, additional studies to reveal single nucleotide polymorphisms, epigenetic and transcription changes, novel mutations and protein stability, will surely contribute to establish a firm mechanistic basis of AQPs contribution to the etiopathogenesis of MetS.

Agradecimentos

Ainda que esta dissertação seja uma prova individual de esforço e dedicação, é também resultado de várias contribuições. Por essa razão, quero agradecer:

À Professora Doutora Graça Soveral, que sem me conhecer me recebeu no seu laboratório e me fez sentir em casa, pelo papel fundamental que teve no decurso do desenvolvimento desta dissertação, bem como todo o apoio e ajuda que me deu ao longo deste tempo.

À Professora Doutora Irene Rebelo, por ter aceite fazer parte deste projeto, representando a Faculdade de Farmácia da Universidade do Porto.

À Professora Doutora Joana Miranda, pela orientação e aconselhamento durante o trabalho prático realizado no seu laboratório.

À Doutora Paula Lopes, por ter estado presente durante estes anos, por transmitir calma e confiança, por incutir profissionalismo, exigência e rigor e por ser um exemplo profissional, tendo simultaneamente sempre uma palavra amiga. Por permitir que fizesse parte dos seus projetos e por me ajudar e apoiar nos meus!

Ao “triângulo”, que se completa com a Andreia Mósca e a Cláudia Rodrigues, companheiras de laboratório, pela amizade, ajuda e preocupação ao longo de todos estes anos. Podemos seguir caminhos diferentes, mas esta ligação jamais se quebrará ☺!

À professora Doutora Teresa Moura, pelo entusiasmo contagiante, pelas aulas de introdução às “permeabilidades” e pelos conselhos experientes.

À Professora Doutora Rita Castro e a Madalena Barroso, por me terem ensinado, ajudado e apoiado no projeto das células endoteliais.

À minha Rutezinha, à Catarina, à Ana Sofia, ao Duarte, ao António e ao André, por terem feito parte do grupo BiolTransp, pois aprende-se tanto a ensinar! Espero que tenham aprendido alguma coisa comigo!

Aos residentes do Salão Nobre “Doutora Joana Marto”: à Doutorazinha, à Madalena, ao Sérgio, à Joaninha, ao Pedro, ao Bernardo e à Diana, por estarem dispostos a ajuda sempre que precisei, pelo apoio em dias menos bons, por me fazerem rir, pelos momentos de distração, por estarem presentes!

Ao Pedro Rodrigues, por me ter ajudado na resolução de tantos problemas e pela sua, (sempre!) boa disposição.

À Leire, pela energia e alegria contagiante, pela ajuda e por todos os ensinamentos, pelos momentos de distracção e diversão!

Al Pablo, mi especial agradecimiento, por haberme aceptado en su laboratorio, por toda la ayuda en el proyecto! A mis colegas de Murcia: Diego, Mari Carmen, Helios, por

toda la ayuda en el desarrollo del trabajo experimental; y Ana T, Carlos, Ana I y María por toda la ayuda en el laboratorio, por los momentos de relajación, por ayudarme a buscar piso, por la amistad!

À Dani, a portuguesa que fez com que a estadia em Múrcia nunca me tenha mostrado a verdadeira dificuldade de estar fora de casa, do país, de me sentir sozinha ou sem companhia. Pelos momentos de discussão científica, pelos ensinamentos e pelos momentos de diversão!

Ao Professor Doutor Nuno Santos, que permitiu que integrasse o seu grupo para fazer os ensaios de AFM. Ao Doutor Peter Eaton e à Constança pela colaboração nos ensaios de AFM. À Patrícia Silva, Patrícia Carvalho e Mário Felício por terem estado disponíveis para ajudar sempre que necessário.

Ao Professor Manuel Bicho, por me ter recebido no Laboratório de Genética, Instituto Egas Moniz, por ter permitido que fizesse parte do meu trabalho no seu laboratório e por partilhar o seu imensurável conhecimento e entusiasmo pela bioquímica!

À minha Carol, por me fazer sentir “em casa” quando me mudei temporariamente para o Laboratório de Genética, por ter estado sempre disponível para me tirar imediatamente qualquer tipo de dúvida, pela companhia e pelos conselhos! À Andreia Matos, pela ajuda e aconselhamento durante a minha estadia no Laboratório de Genética. À Joana Ferreira pela revisão do capítulo dos polimorfismos e pela sua boa disposição contagiante!

A Profesora Doctora Marta Camps y Profesora Doctora Anna Gumà, por haberme recibido en su laboratorio en Barcelona con todo el apoyo necesario!

A Fran, por haberme ayudado de forma incansable en la adquisición del modelo de "browning" y por todas las discusiones científicas. ¡A Núria y Freddy, por haber ayudado siempre que lo necesitaba!

À Ana, a minha irmã, ironicamente símbolo de que a vida não é só trabalho, por ter vindo passar comigo inúmeros fins de semana, nos quais a ciência e o trabalho eram postos de lado!

Aos meus pais, que à sua maneira me ensinaram a ser independente, não desistir e lutar pelos meus objetivos, pelos sacrifícios que fizeram para que chegasse aqui!