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Leukocyte AQP3 and AQP9 in human sepsis: cellular and Machine Learning-driven approaches in studying their pathophysiological and prognostic relevance

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List of abbreviations:

AQP	Aquaporin
APL	Acute Promyelocytic Leukemia
ATRA	All-Trans Retinoic Acid
AUROC	Area Under the Receiver Operating Characteristic Curve
Akt 1/2/3	RAC-alpha/beta/gamma serine/threonine-protein kinase
β-Catenin	Beta-catenin
CD64	Cluster of Differentiation 64
Chk-2	Checkpoint kinase 2
c-Jun	Transcription factor AP-1 (c-Jun)
CREB	cAMP response element-binding protein
CRP	C-Reactive Protein
DAMPs	Damage-Associated Molecular Patterns
DMSO	Dimethyl Sulfoxide
dNB4	Differentiated NB4 cells
EGFR	Epidermal growth factor receptor
EMR	Electronic Medical Records
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinases 1 and 2
Fgr	Gardner-Rasheed feline sarcoma viral oncogene homolog
GSK	3 alpha/beta – Glycogen synthase kinase-3 alpha/beta
HSP27	Heat shock protein 27
HSP60	Heat shock protein 60
ICU	Intensive Care Unit
IL-10	Interleukin-10
JNK 1/2/3	c-Jun N-terminal kinases 1, 2, and 3
Lck	Lymphocyte-specific protein tyrosine kinase
LncRNA	Long Non-Coding RNA

LPS	Lipopolysaccharide
Lyn	Tyrosine-protein kinase Lyn
MD2	Myeloid Differentiation Factor 2
MEWS2	Modified Early Warning Score 2
ML	Machine Learning
MSK1/2	Mitogen- and stress-activated protein kinases 1 and 2
NF-κB	Nuclear Factor Kappa Beta
NLRP3	NOD-, LRR- and Pyrin Domain-Containing Protein 3
p38α	p38 mitogen-activated protein kinase alpha
p53	Tumor protein p53
p70S6K	Ribosomal protein S6 kinase beta-1
PBMC	Peripheral Blood Mononuclear Cells
PAMPs	Pathogen-Associated Molecular Patterns
PDGFRβ	Platelet-derived growth factor receptor beta
PLCγ1	Phospholipase C gamma-1
PCT	Procalcitonin
PRAS40	Proline-rich AKT1 substrate 1
PRRs	Pattern Recognition Receptors
PTX-3	Pentraxin-3
Pyk2	Protein tyrosine kinase 2 beta (FAK2)
qPCR	Quantitative Polymerase Chain Reaction
qSOFA	Quick Sequential Organ Failure Assessment
ROC	Receiver Operating Characteristic
RSK1/2/3	Ribosomal S6 kinase 1, 2, and 3
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
sTREM-1	Soluble Triggering Receptor Expressed on Myeloid Cells-1
SIRS	Systemic Inflammatory Response Syndrome
SOFA	Sequential Organ Failure Assessment
Src	Proto-oncogene tyrosine-protein kinase Src

STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STAT3	Signal transducer and activator of transcription 3
STAT5a/b	Signal transducer and activator of transcription 5a/b
STAT6	Signal transducer and activator of transcription 6
suPAR	Soluble Urokinase-Type Plasminogen Activator Receptor
TLR4	Toll-like Receptor 4
TNF-α	Tumor Necrosis Factor Alpha
WNK-1	WNK lysine-deficient protein kinase 1
Yes	Tyrosine-protein kinase Yes

I.1.GENERAL ABSTRACT

Sepsis is a multifaceted pathology defined as a severe, potentially fatal, organic dysfunction caused by an inadequate or dysregulated host response to infection. Despite the growth in clinical care and knowledge, the in-hospital mortality rate related to sepsis remains above 10%, while in cases of septic shock, its most severe complication characterized by multiple organ failure, it exceeds 40%.
The precise mechanisms driving sepsis and systemic inflammatory responses often involve aberrant immune cell function, excessive production of pro-inflammatory cytokines, and impaired resolution of inflammation. Emerging evidence during the last years highlights the role of Aquaporin channel proteins in modulating key processes during inflammation, including immune cell maturation, migration, phagocytosis, and cytokine release, leading to a gain of interest about AQPs involvement in inflammatory disorders.
Aquaporins (AQPs) are a family of membrane channel proteins expressed in all body districts where they play important roles in health and disease (Agre, 2004). Humans possess 13 distinct AQPs (AQP0-12) grossly subdivided into three groups, *orthodox aquaporins* (AQP0, AQP1, AQP2, AQP4, AQP6, and AQP8), *aquaglyceroporins* (AQP3, AQP7, AQP9, and AQP10) and *unorthodox aquaporins* (*superaquaporins*; AQP11 and AQP12), depending on their molecular selectivity and evolutionary pathway. This classification is not exhaustive, and some members are also referred to as *peroxiporins* (AQP1, AQP3, AQP5, AQP8, AQP9, AQP11) and as *ammoniaporins* (AQP1, AQP3, AQP6-AQP9) since they also allow movement of hydrogen peroxide and ammonia, respectively, in addition to water and other molecules.
While most of the work on AQPs in septic conditions has been conducted *in vitro* or in animal models, little has been done by using *ex vivo* human samples from patients. Moreover, assessing their differential expression might be of translational value in developing novel therapeutical strategies, as well as defining them as easily analyzable biomarkers to screen for diseases.
The PhD work was carried out following three main aims sharing all the same common points to evaluate the involvement of AQPs in inflammatory disorders.
The first main aim of this doctoral work was to investigate the expression of AQP3 and AQP9 in circulating human white blood cells. Additionally, we aimed at characterizing our novel, custom-designed polyclonal antibody, which will be used in flow cytometry analyses to identify and screen the sub-population of blood cells expressing AQP9.
The second main aim was to assess the relevance of AQP3 and AQP9 as diagnostic and/or prognostic biomarkers in both PBMC and neutrophils in human sepsis by using bio-statistical and machine-learning approaches. We detected a significant increase in AQP3 expression in the PBMC of septic patients while no significant differences were found regarding AQP9 expression in neutrophils. Furthermore, statistical analyses correlated the expression levels of these AQPs with critical clinical parameters, including SOFA and MEWS scores, blood lactate levels, and procalcitonin, which are widely used in routine diagnostics. These findings were then integrated into a Machine Learning (ML) model, employing a Random Forest classifier, to develop a predictive tool for early sepsis detection and patient mortality prognosis.
To test AQPs as possible pharmacological targets to treat inflammatory disorders such as sepsis, the third main aim was that of characterizing the expression of AQP9 in NB4 cells, a human Acute Promyelocytic Leukemia (APL) cell line that in future studies will be used as a model of mature human

neutrophil granulocytes to investigate the role, biophysical properties, modulation and pharmacological relevance of AQP9 in neutrophils.
The differentiation of NB4 with the combined effect of ATRA and G-CSF led to a significant increase in AQP9 expression, mimicking human derived neutrophils phenotype. Moreover, the phosphorylation profiling of cell signaling pathway proteins suggested (by selective gating of AQP9 upon LPS, TNFα, IL-6 and fMLP stimulation) a role for AQP9 in regulating key processes such as neutrophil migration and adhesion as well as neutrophil-mediated immune responses. Defining the NB4 cell line as a suitable cell model for investigating AQP9 involvement in immune cells functions.
The results obtained and the related discussions are described in each chapter composing section II, while general conclusions on the whole doctoral work are discussed in Section III.

I.2.GENERAL INTRODUCTION

I.2.1. Sepsis pathophysiology: from systemic inflammatory response syndrome to multi-organ disfunction

Sepsis is a life-threatening condition characterized by physiological and biochemical abnormalities caused by non-sterile infections, approximately 50 million of people are affected by sepsis every year and the estimation led to 11 million deaths caused by the disease (Rudd et al. 2020).
Systemic inflammatory response syndrome definition has been debated for decades, with the current definition being *"the systemic inflammatory response to a variety of severe clinical insults"* manifested by at least two abnormalities across: hypothermia or hyperthermia, tachycardia, tachypnea or hyperventilation, leukopenia or leukocytosis (Robertson and Coopersmith 2006).
Despite a clear definition of the disease, SIRS is characterized by a dysregulated immune response to a sterile infection, where both innate and adaptive immune system play a role.
The first line of defense is represented by the innate immune system, where innate cells, like neutrophils and macrophages recognize specific molecules, known as Pathogen-associated molecular patterns (PAMPS) through their Pattern recognition receptors (PRRs), upon recognition these cells generate a response which usually end with the clearance of the invading bacteria through phagocytosis.
The specific recognition between PRRs and PAMPS leads to the activation of signaling cascades which result in cytokines production.
Toll-like receptor 4 (TLR4) is the best characterized PRR binding LPS, a component of Gram-negative bacteria cell wall. Upon LPS binding, in presence of proteins like CD14 and MD2, TLR4 initiates the signaling cascade that leads to the recruitment of kinases such as IRAK-4, that phosphorylates the IκB protein, with consequent activation and nuclear translocation of the NF-κB transcription factor, allowing the expression of pro-inflammatory mediators.
Despite the well-established role of TLR4 signaling pathway in sepsis pathophysiology, it also plays a role in cases of non-infectious SIRS, where evidences have shown that it might be relevant to activate typical pathways of SIRS (Robertson and Coopersmith 2006).
Patients with SIRS are characterized by a hyperinflammatory response and its severity depends on both intrinsic host factors and extrinsic factors. Host factors include age, gender, genetics and co-morbidities, while host-independent factors include the virulence of the pathogen and/or the magnitude of the injury.
SIRS pathology is characterized by a multi-hit course, after the initial hyperinflammatory response caused by an infection or injury, a second one can occur leading to an elevation of pro-inflammatory cytokines levels.
In cases where the insult does not resolve quickly or in cases of co-morbidities, the hyperinflammatory state is followed by a long hypo-inflammatory state which can last from weeks to months leaving the patients with lowered immune defenses. In cases where the hypo-inflammatory state is not reversed and the immune system is compromised, patient progresses through organ dysfunction which leads to the patient's death.

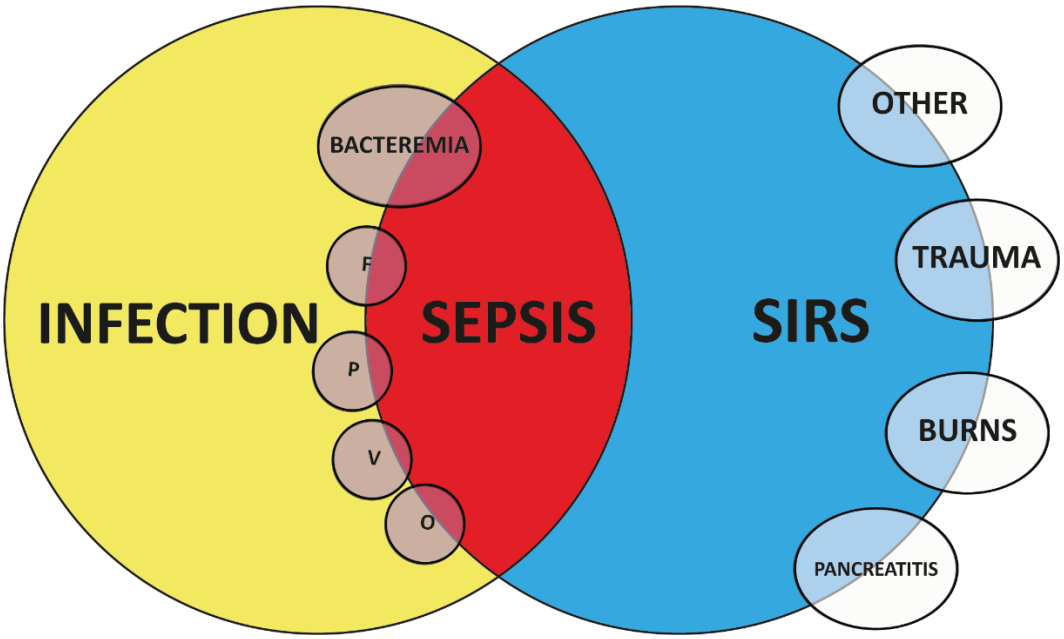


Figure 1 – **Schematic representation of Sepsis and SIRS etiological agents.** F: Fungemia; P: Parasitemia; V: Viremia; O Other

Sepsis is a sub-set of SIRS syndromes and during the last decades, different definitions of sepsis have been proposed, with the latest being the definition of the Third International Consensus (Sepsis-3) which defines sepsis as a *"organ dysfunction caused by a dysregulated host response to infection"* (Singer et al. 2016) (Fig. 1).

Approximately 50 million people are affected by sepsis every year and the estimation led to 11 million deaths caused by the disease (Rudd et al. 2020).

Its most severe complication, known as septic shock, is characterized by circulatory, cellular and metabolic abnormalities, with an intra-hospital mortality rate which reaches 60% (Vincent et al. 2019).

The disease shares the same inflammatory activation pathways with the SIRS, where, after the initial host-microbial interaction, a disseminated activation of immune responses occurs (Cohen 2002; Chakraborty and Burns 2025) aimed at coordinating defensive responses involving both humoral and cellular components. Immune cells play a pivotal role in releasing pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, IL-12, IL-15 and IL-18, with the IL-1 and TNF-α being the first to be released within 30-90 minutes after the exposure to the microbial LPS , activating the release of secondary cytokines and reactive oxygen species (ROS) (Dinarello 1997; Schulte, Bernhagen, and Bucala 2013).

Major key players in the activation and release of pro-inflammatory cytokines are inflammasomes, complexes of cytoplasmic proteins which mediate the host immune response against DAMPS (damage-associated molecular patterns) or PAMPS (pathogen-associated molecular patterns), these complexes consist of three main components: a sensor protein, an adapter protein ASC (apoptosis-associated spot-like protein containing CARD) and the effector protein, the pro-caspase 1 (He et al. 2023).

Based on different sensors protein, several inflammasomes have been identified such as NLRP3, NLRC4 and NLRP1, with the NLRP3 being the most investigated inflammasome (Agostini et al. 2004), due to its ability of being activated by a wide variety of stimuli (Yang et al. 2019), via a multi-step process known as "canonical activation".

The first step, also known as "priming", is triggered by the recognition of various PAMPS or DAMPS through PRRs (such as TLRs) and the subsequently activation of NF-κB which up-regulates the transcription of inflammasome components (Afonina et al. 2017; Xing et al. 2017), while the second step, whose exact mechanism is still under investigation (He et al. 2023), occurs upon the recognition of the stimulus by the Inflammasome, triggering the recruitment of ASC protein and the caspase (Fig. 2) (Burns, Martinon, and Tschopp 2003; Halle et al. 2008; Hornung et al. 2008).
NLRP3 consist in a three part structure: a pyrin domain (PYD), a nucleotide-binding NACHT domain and a leucine-rich-repeat (LRR) domain (Strowig et al. 2012), and upon activation, NLRP3 binds to the ASC adapter protein, combining then with the pro-caspase 1, leading to the formation of the active NLRP3 inflammasome through caspase-1 self-cleavage and activation. Activated NLRP3 inflammasome is then able to promote the maturation and release of pro-inflammatory cytokines such as IL-1β and IL-18 (Broz and Dixit 2016) (Fig. 2).

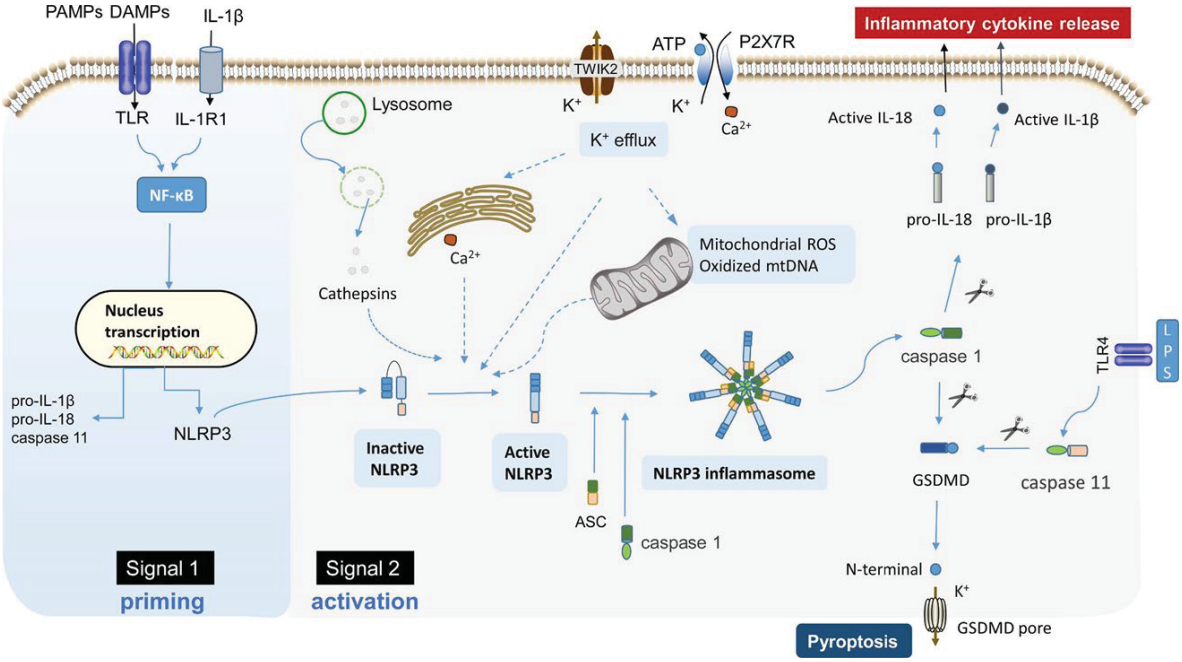


Figure 2 – Schematic representation of NLRP3 inflammasome activation pathway. The priming process (signal 1) is triggered by the recognition of PAMPS or DAMPS via Pattern recognition receptors which activate the transcription of inflammasome components and pro-inflammatory cytokines. The activation process (signal 2) occurs after the recognition of the stimulus, followed by the assembly and activation of the NLRP3 inflammasome leading to the maturation and release of pro-inflammatory cytokines. From (He et al. 2023)

Pro-inflammatory cytokines released after microbial exposure are also the cause of the excessive coagulation reported in septic patients, with the 30-50% of the affected patients presenting a disseminated intravascular coagulation (Levi and Ten Cate 1999), on top of this release of cytokines, in septic conditions, downregulation of anticoagulant proteins (antithrombin and protein C) have also been reported (Okajima 2001).
Upon activation of pro-inflammatory pathways, to restore the immunological homeostasis, anti-inflammatory cytokines are released, such as soluble receptors for TNF and IL-1, together with the release of cytokines inhibitors, septic patients develop lymphocyte apoptosis (Hotchkiss et al. 1999), that leads to a general condition of immunosuppression characterized by a T-cell hypo-responsiveness, principal cause of the *"inadequate host response against the infection"* (Cohen 2002).

Several reports have also defined a role of genetics in the pathogenesis of sepsis, particularly, a mutation in the TLR4 loci has been reported to be associated with an increased susceptibility to Gram-negative bacterial sepsis (Lorenz et al. 2002).
The most common condition which sepsis leads to is represented by multiple organ failure, a multifactorial process still incompletely understood, characterized by tissues hyperfusion and hypoxia, resulting from a widespread deposition of fibrin causing microvascular occlusions compromising the oxygenation (Cohen 2002). Moreover, tissue infiltrated cells (neutrophils among all) damage tissues by releasing lysosomal enzymes and reactive oxygen species (Zhang et al. 2023).
Advancing our understanding of the molecular pathways involved, including the roles of PRRs, cytokines, and inflammasome activation, is essential for developing targeted therapeutic strategies aimed at modulating immune responses and lowering the high mortality rate which sepsis and septic shock carries out.

I.2.2. Artificial Intelligence in Clinical Care: Enhancing Outcomes in Intensive Care Units (ICUs)

The past century has been characterized by a massive increase in computational power provided by computers and a massive increase in connectivity provided by the advent of internet.
The term Artificial intelligence (AI) refers to a computer system capable of developing intellectual processes characteristics of humans such as: discover, reason and learn from past activities.
AI computers are capable of performing tasks that normally were requiring human intelligence to be performed (Gutierrez 2020).
Capabilities of AI have been exploited in medicine applying it to drug discovery, personalized diagnosis and treatments, bioinformatic analysis, medical imaging as well as sorting and discerning diseases patterns in massive dataset formed by the electronics medical records (EMR).
Application of computational-based systems in clinical care is not a new concept, different systems have been proposed for monitoring ICU patients, managing mechanically ventilated patients (Grossman, Hew, and Aberman 1983; Ohlson, Westenskow, and Jordan 1982) and manage arterial oxygenation (Henderson et al. 1991).
All these early computer-based systems relied on logical evaluation of parameters to execute instructions, and this represented a limitation in a real-world environment characterized by a multiplicity of different parameters that needed evaluation.
Artificial intelligence represents a huge improvement when compared to the old systems, since its capabilities have been compared to the human brain, being able to adapt and respond to different stimuli as a human brain would do, learning from the data (Gutierrez 2020).
A subset of AI is Machine Learning (ML), where machines can learn and extract knowledge from the available data. These approaches combine statistics and computer science to devise algorithms capable of learning through statistical relations. Algorithm are based on two different learning structures: supervised learning and unsupervised learning (Gutierrez 2020) (Fig. 3).

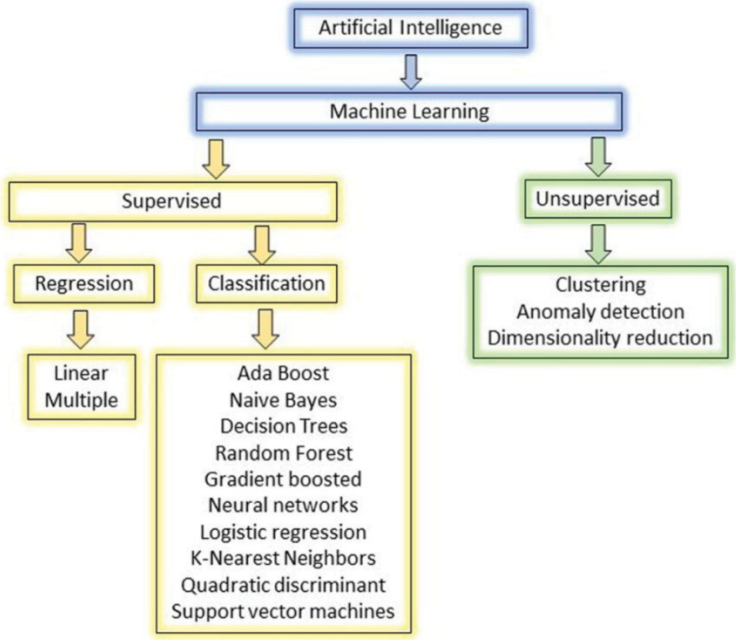


Figure 3 – Graphical representation of the two major branches of Machine Learning with the typical algorithm involved. From (Gutierrez 2020)

Supervised learning methods are used to develop algorithms capable of predicting a single output when provided with a single input, but it depends on human effort to build large enough datasets to train and validate the Algorithms.
Supervised algorithms divide also into two main learning algorithms, regression learning, useful in continuous variables datasets, or classification learning, used in cases of mixed datasets typical of hospitals recordings.
Unsupervised learning, on the other hand, does not require any effort from the researcher, since the algorithm is able to extract knowledge and generate relationships on its own from a given dataset, these approaches are usually preferred when exploratory analysis are needed to give insights into the EMR (Gutierrez 2020).
Due to its capabilities of images recognition, supervised learning has been extensively used in radiology (Hosny et al. 2018) and histopathology (Litjens et al. 2016), to automate pattern recognition in images.
Although Machine Learning is slowly gaining interest in ICUs departments, several studies have already been performed evaluating its performance in risk stratification in critically ill patients.
By using electrocardiograms (EKG) records, Yoon and colleagues developed a method to predict patients instability in the ICU through a combined approaches of logistic regression and random forest algorithms, with an accuracy of 0.81 and an AUC of 0.87 (Yoon et al. 2019).
A recent study used a random forest classifier to predict the occurrence of sepsis and septic shock using over 200.000 electronic medical records as a training dataset, although the algorithm has been proven to be specific, with a specificity of 98%, unfortunately lacked of sensitivity with a value of 26%, highly limiting its application in ICUs (Giannini et al. 2019).
The greatest challenge that needs to be tackled when a clinical machine learning model is devised is the identification of the correct algorithm to use for the classification and the datasets used for its

validation, since the accuracy of a machine learning algorithm is calculated based on its ability to correctly predict a blind validation dataset.
Despite the potential benefits deriving from AI application to hospitals, there are still challenges to its enrollment, since implementing a computing system for collecting, storing and sharing EMRs and sensitive health data remains a challenge on different levels (Lee and Gostin 2009), since the deployment on large scale of these algorithms need legal certifications, and represent an economical burden for hospitals.
Due to the high heterogeneity of symptoms, in sepsis cases, novel and robust biomarkers have been proposed, in this context, AQPs have been proposed as novel promising biomarkers which could be implied in AI-based screening routines.

I.2.3. Human Aquaporins: structure, cellular and subcellular localization and functions

Aquaporins are a family of membrane protein channels known to allow transport of water and small neutral solutes across biological membranes (Agre 2004).
In mammals, 13 AQPs have been reported, from AQP0 to AQP12 which share the same structural characteristics (Li and Wang 2017), despite significant differences in primary structure, Aquaporins have a highly conserved structure across isoforms.
Each Aquaporin is constituted by 6 transmembrane α-helices, connected by three extracellular loops (A,C and E) and two intra-cellular loops (B and D) (Agre et al. 2002) (Fig. 4A).
The channel is formed as a membrane pore delimitated by the six transmembrane domains where half of the transmembrane helices formed by the B and D loops fold into the channel, assuring substrate selectivity (Sui et al. 2001).
Water molecules are allowed to pass singly through the pore due to a particularly conserved structural feature known as aromatic/Arginine constriction site (ar/R) located in the extracellular side of the channel with a diameter of 3 Å.
The internal surface of the pore formed by AQPs is hydrophobic, except a single line of amino acids, known as NPA which use their oxygen and nitrogen atoms to allow the formation of hydrogen bonds that act as a guide to allow water and small neutral molecules to cross the channel (Tani et al. 2009) (Fig. 4B).
Each Aquaporin is formed by 4 identical monomer and each monomer acts as an independent channel (Agre et al. 1993) (Fig. 4C).

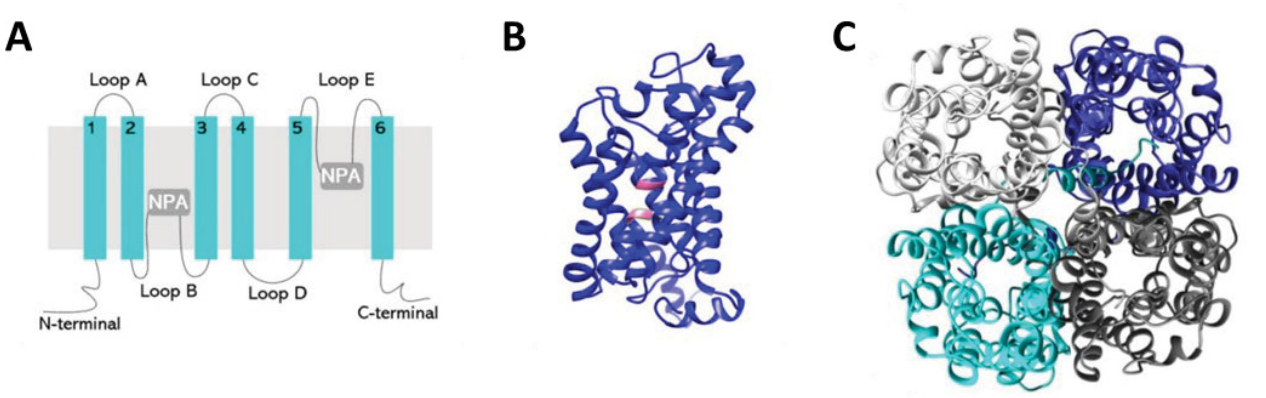


Figure 4 – **Representation of AQP three-dimensional structure using UCSF Chimera software.** (A) Representation of membrane topography, displaying the monomer composed by six trans-membrane helices (1-6) linked by five loops (A-E) highlighting the Asparagine-Proline-Alanine motifs embedded in the membrane. (C) Three-dimensional structure of AQP monomer with NPA motifs (pink colored). (D) Top view of the homo-tetrameric structure of the channel. Modified from (da Silva and Soveral 2021)

Based on sequence and phylogenetic analysis, Aquaporins could be divided into three main groups (Gorelick et al. 2006): *orthodox aquaporins* (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) which initially were thought to be permeable only to water (Agre 2004; Verkman 2005); Aquaglyceroporins (AQP3, AQP7, AQP9, AQP10) which are permeable to water and small non-charged solutes as Urea and Glycerol (Agre 2004; Rojek et al. 2008; Verkman 2005); the *superaquaporins* (known also as *unorthodox aquaporins*) AQP11 and AQP12, characterized by a different evolutionary path (Fig. 5-A), whose permeability to water and glycerol is still under investigation (Li and Wang 2017).

This nomenclature, based on phylogenetic bases, has been changed with other subgroups, following the pore selectivity to the solutes in addition to water and/or glycerol, in fact AQP1, AQP3, AQP5, AQP6, AQP8, AQP9 and AQP11 have been referred as *peroxiporins* due to their permeability to Hydrogen Peroxide (Almasalmeh et al. 2014) while, those permeable to ammonia, such as AQP3, AQP4, AQP6, AQP7, AQP8 and AQP9 have also been referred as *ammoniaporins* (Fig.5-B) (Saparov et al. 2007).

It has been reported that some of the Aquaporins are able to mediate the movements of gases such as CO_2 (AQP1, AQP4 and AQP5), Nitric Oxide (AQP1 and AQP4) and oxygen (AQP1 and AQP4) (Herrera, Hong, and Garvin 2006; Saparov et al. 2007; Wang et al. 2007).

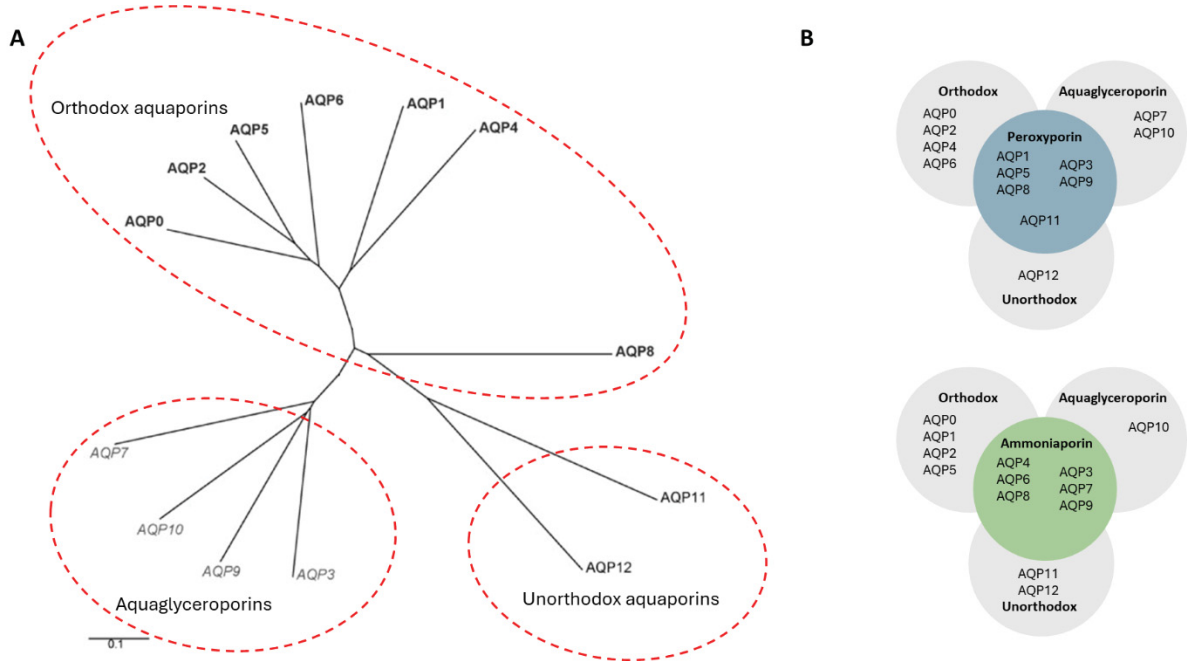


Figure 5 – Representation of phylogenetic division and functional division of human Aquaporins. (A) Orthodox Aquaporins (AQP0, AQP2, QAP5, AQP6, AQP1, AQP4, AQP8) are represented in bold, Aquaglyceroporins (AQP7, AQP10, AQP9, AQP3) are shown in Italic, Unorthodox Aquaporins subfamily (AQP11, AQP12) are circled in the bottom right. Scale bar represents phylogenetic distance. Modified from (Gorelick et al. 2006). (B) Subgroups of the Aquaporins based on their selectivity, peroxiporins (AQP1, AQP3, AQP5, AQP8, AQP9, AQP11) are shown in blue circle while Ammoniaporins are shown in green circle (AQP3, AQP4, AQP6, AQP7, AQP8, AQP9).

The transport mediated by Aquaporins is a mechanism depending on the osmolarity of the solutions across the membrane that can be mediated by the translocation of vesicles containing Aquaporins to and from the membrane and their expression and distribution vary significantly across tissues and cells (Conner, Bill, and Conner 2013).

Different studies have investigated and reported the spatial and temporal redistribution of AQP2 from the membrane of intracellular vesicles to the apical membrane of the collecting duct under the action of Arginine Vasopressin (AVP) bind to the Vasopressin receptor 2 (V2R) (Nedvetsky et al. 2009).

Also the AQP3 and AQP4 translocation to the basolateral membrane mediates the reabsorption of water from the lumen of the kidney collecting duct (Conner, Bill, and Conner 2013).

Other AQPs share the same mechanism, such as AQP1 in cholangiocytes which translocate to the plasma membrane when the Secretin binds to specific G protein-coupled receptors (Marinelli and LaRusso 1997).

The wide distribution and the abundance of Aquaporins provide selective pores for the rapid movement of water and other solutes across membranes and through molecular dynamic simulations, open and closed states have been proposed as gating mechanism for human Aquaporins (Janosi and Ceccarelli 2013).

I.2.4. Aquaporin-3 and Aquaporin-9 pathophysiological relevance

AQP3 and AQP9 are key players in many physiological conditions and their dysregulation and/or dysfunction is related to several diseases (Madeira, Moura, and Soveral 2016).

AQPs are expressed in many fluid-transporting tissues, such as renal tubules and glandular epithelia, as well as in non-fluid-transporting tissues, such as epidermis, adipose tissue, and astroglia (Verkman 2005). AQP3 is expressed at various sites, playing several roles, both in physiological and pathophysiological conditions. It is widely expressed on the plasma membrane of the principal cells of the renal collecting duct and on epidermal keratinocytes of the skin, where it facilitates the transport of water and glycerol (Tricarico et al. 2022), but also in the epithelial cells of the eye, being involved in tear formation and external humidification (Tran et al. 2014), and in the digestive (Liao et al. 2021) and respiratory system (Liu et al. 2007).

In the skin, AQP3 plays a role in the proliferation, migration, differentiation and survival of keratinocytes, as well as its role in skin properties and functions such as hydration, water retention, wound healing and barrier repair (Tricarico et al. 2022).

Post-translational modifications such as glycosylation (Qin et al. 2011) may regulate the subcellular localization of AQP3 as occurs for AQP2 in the principal cells of renal-collecting ducts translocation to the plasma membrane. Phosphorylation may be another post-translational modification regulating the subcellular localization of AQP3, but no evidence supports this eventuality.

The importance of AQP3 in skin is highlighted by its dysfunctions observed in human skin diseases. A study based on non-melanoma skin cancer cells characterized by excessive proliferation, reports decreased levels of AQP3 immunoreactivity in proliferating regions when compared to normal human epidermis (Voss et al. 2011).

The knockdown of AQP3 in human keratinocytes decreased glycerol uptake, scratch wound healing and fetal bovine serum-induced migration in vitro (Hara-Chikuma and Verkman 2008). It has been suggested that the effect of AQP3 in skin keratinocyte migration relates to the facilitation of water movement instead of the transport of glycerol (Hara-Chikuma and Verkman 2008).

In human keratinocytes AQP3 upregulation increased glycerol uptake and cell growth, while its silencing inhibited their proliferation in response to the cytokine CCL17 (Nakahigashi et al. 2011). Moreover, a body of evidence indicates that the facilitated transport of hydrogen peroxide through AQP3 is relevant in cell migration (Hara-Chikuma et al. 2012; Miller, Dickinson, and Chang 2010).

Studies conducted on *Aqp3* knockout mice models ($Aqp3^{-/-}$) highlighted reduced tumor formation when submitted to the topical application of a carcinogen followed by a treatment with a tumor promoter. Moreover, were shown to have significantly reduced epidermal glycerol, glucose and ATP contents, indicating that the absence of AQP3 results in impaired cellular metabolism (Hara-Chikuma and Verkman 2008).

AQP3 has also been found to promote epithelial-mesenchymal transition in gastric cancer cells (Chen, Wang, et al. 2014). AQP3 is also expressed on the basolateral membrane of the principal cells of the collecting duct and affects renal functions. Evidences confirm that AQP3 deficiency in mice reduces the water permeability of the collecting ducts and consequently impairs the ability to concentrate urine, after increasing its production (Ma et al. 2000).

Lastly, AQP3 has also been reported in human Kupffer cells and appears to be implicated in liver disease and its regeneration processes (Gregoire et al. 2015).

Several roles for AQP3 in promoting cell proliferation have also been reported in several cancers (Marlar et al. 2017).
Strong *AQP9* mRNA expression has been found in liver and peripheral leukocytes in humans (Ishibashi et al. 1998; Kono et al. 1999) while AQP9 immunoreactivity has been reported in many other tissues, however its expression in some tissues is still debated due to false positive identification.
In the immune system the AQP9 is expressed in neutrophils, macrophages, $CD8^+$ T cells, dendritic cells where it plays several roles, it has been reported that the water influx through AQP9 leads to morphological and functional changes in neutrophils during the F-Actin polymerization (Matsushima et al. 2014).
In murine neutrophils AQP9 is required to develop the sensitization during murine cutaneous-acquired immune responses via regulation of cell function (Moniaga et al. 2015).
In human primary monocyte-derived macrophages, AQP9 was shown to be upregulated and redistributed to the leading and trailing regions upon macrophages infection with *Pseudomonas aeruginosa*, promoting the phagocytosis of bacteria (Holm, Karlsson, and Vikstrom 2015).
In murine memory $CD8^+$ T cells, IL-7 induces the expression of aquaglyceroporin AQP9 that facilitate the entry of glycerol required for the fatty acid esterification and triglyceride (TAG) synthesis and storage, for their long-term survival (Cui et al. 2015).
Recently it has been reported that AQP9 is involved in macrophages M2 polarization in the progression of kidney renal clear cell carcinoma (Jing et al. 2021), with previous studies indicating that AQP9 in functionally involved is murine bone marrow dendritic cell maturation in response to inflammatory stimuli (De Santis et al. 2018) and in LPS-induced endotoxemia (Tesse et al. 2021), a role in neutrophil infiltrations has been recently reported in a study using a murine model of polymicrobial infection.
These studies highlighted the need of AQP9 to activate the NF-κB transcriptional factor and its ablation reduced the expression of NLRP3 inflammasome in heart and kidney, shedding new lights on signaling pathways through which AQPs might modulate the inflammatory processes (Mohammad et al. 2022).
Less defined is the role played by AQP9 in other tissues where this AQP is expressed. In the stratum granulosum of the epidermis AQP9 was suggested to represent a transcellular route for the movement of biologically relevant solutes, such as glycerol and urea, into and out of the skin, being involved in the terminal differentiation of keratinocytes (Tricarico et al. 2022).
In the principal cells of the epididymis and vas deferens, AQP9 appears to be implicated in sperm concentration and storage (Pastor-Soler et al. 2001).
Moreover, upregulations of glycerol permeable AQPs, such as AQP3 and AQP9, might be relevant in inflammatory diseases due to the alterations in metabolic processes during the pathology course, these altered metabolic pathways involve glycolysis and fatty acid metabolism (Koos et al. 2024), crucial for meeting the elevated energy demands imposed by the disorder (Wang et al. 2024), in addition, the glycerol influx through AQPs could enhance the p38 signaling pathway which is associated to the upregulation of glycolysis and nitric oxide production, allowing immune cells to meet the energy demands caused by noxious agents (Dai et al. 2020).
Chronic liver injury (CLI), a common disease which is harmful to human health , usually manifests as hepatic steatosis and secondary chronic steatohepatitis, and are form of diseases that can develop to liver fibrosis, cirrhosis and further, to hepatocellular carcinoma (HCC) if not treated in time (Cheng

et al. 2021). Liver cancer represents the sixth most common oncologic condition and the second-leading cause of death from malignant neoplasm around the globe (Liu et al. 2019).
In human liver AQP9 is involved in several disorders such as steatosis (Gu et al. 2015), hepatocellular carcinoma (HCC) (Liao et al. 2020), non-alcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) (Rodriguez et al. 2014)
These findings highlight the potential of AQP9 as a clinical biomarker due to its alteration in clinical conditions such as cancer progression and inflammatory disorders. In human leukocytes it has been reported that AQP9 expression increases after intravenous or in vitro LPS stimulation (Talwar et al. 2006; Vassiliou et al. 2013), moreover, its expression levels have been found increased in activated polymorphonuclear leukocytes in subject affected by systemic inflammatory response syndrome (SIRS) (Matsushima et al. 2014) and infective endocarditis (Thuny et al. 2012). In addition, patients with SIRS showed increased AQP9 levels in neutrophils compared to healthy donors control subjects (Matsushima et al. 2014)
Also in leukocytes, AQP9 has been found on the cell edges, most probably to facilitate motility, lamellipodium formation and cell volume changes to allow these cells to migrate toward chemoattractant (Loitto et al. 2002; Loitto et al. 2007).
AQP9 has also been proposed as a possible pharmacological target in human acute promyelocytic leukemia (APL) cell lines such as NB4 and HT93A through arsenic-trioxide treatments, since AQP9 has been reported to be permeable to arsenite (Iriyama et al. 2013).
Clear cell renal cell carcinoma (CCRCC) biopsies showed and increased transcriptional and proteomic AQP9 expression, with transcriptional levels associated with aggressive progression, poor survival and immune infiltration (Xu et al. 2019), leading to the hypothesis that AQP9 is an oncogene and a promising prognostic marker in CCRCC.
AQP9 has also been recently demonstrated to play a key role in the pathophysiology of sepsis, since the RG100204, a potent and selective inhibitory compound for AQP9, reduced the septic cardiomyopathy and multi-organ failure in a cecal ligation and puncture (CLP) induced murine model of polymicrobial infection (Mohammad et al. 2022).
These results suggest that AQP9 might be a novel drug target in polymicrobial sepsis and, at the same time, a novel and valuable biomarker of this pathological condition.
In polycystic ovary syndrome (PCOS), decreased AQP9 expression in the granulosa cells was associated with hyper-androgenism (Qu et al. 2010; Song et al. 2021), influencing the maturation of PCOS follicles (Song et al. 2021).
In gestational diabetes mellitus, placentas from cesarean delivery at term showed higher expression of AQP9 compared to normal placentas (Vilarino-Garcia et al. 2016).
In preeclampsia, serum AQP9 was increased from the onset of the disease compared to healthy women (Damiano, Zotta, and Ibarra 2006; Olmez, Oglak, and Gedik Ozkose 2022).
The analysis of the area under the ROC curve (AUC) demonstrate that the serum AQP9 value could be considered a stable biomarker in preeclampsia diagnosis by depicting the inflammatory state of the patient (Olmez, Oglak, and Gedik Ozkose 2022).
In tubal ectopic pregnancy patients, the fallopian tubes showed lower expression levels of AQP9 than in healthy subjects (Ji et al. 2013).
AQP9 activity as lactate transporter makes this channel essential for primary spermatocytes and for maturation of haploid germ cells since it is a key metabolite in the process. Human varicocele testis

biopsies showed AQP9 downregulation, leading to lactate deprivation with subsequent hypo-spermatogenesis (Arena et al. 2011).
Altogether, Aquaporins might reveal relevant on different levels of inflammatory disorders, from the onset to the clearance of the infections, supporting immune cells functions being involved in metabolic and inflammatory pathways as well as stabilizing cells shape changes allowing immune cells migration.

I.2.5. Aquaporins in immune system

The molecular events which occur in immune cells during inflammation are complex and every protein plays a key role, in the recent years, Aquaporins (AQPs) have been proposed as key player in immune cell physiological functions and inflammatory response (da Silva and Soveral 2021).
AQPs are protein channels which facilitate the transport of water and small non charged solutes across biological membranes, driven by osmotic and solute gradients (Agre 2004).
The cells constituting immune system, mostly neutrophils, lymphocytes and monocytes, can rapidly adapt and act against danger signals, being capable of undergoing through rapid morphological modifications (Maidhof et al. 2014).
Aquaporins involvement in developing inflammatory processes have been proposed due to the detection of several AQPs isoform in both innate and adaptive immune cells, proven by their dysregulation in different human diseases (Ishibashi et al. 1998; Moon et al. 2004).
In the last few years, AQPs are being reported to play a role in specific immune cells processes such as priming and inflammasome activation, trans-endothelial migration and phagocytosis (Holm, Karlsson, and Vikstrom 2015; Holm, Magnusson, and Vikstrom 2016; Jablonski et al. 2004; Rabolli et al. 2014; Zhu et al. 2011).
Recently, studies conducted on cells and animal models, pointed out a role for AQPs, especially AQP3 and AQP9, in NLRP3 inflammasome activation in macrophages, being the fast reswelling in the presence of danger signals a necessary condition to activate the inflammasome and secrete pro-inflammatory cytokines, such as IL-1β and IL-18 (Boyle, Bryant, and Monie 2013; Compan et al. 2012; Schorn et al. 2011).
Moreover, taking in account the peroxiporin activity of AQP3 and AQP9, they might also contribute to the rising in intracellular ROS levels needed for the subsequent inflammasome activation (da Silva and Soveral 2021).
Other than activating the NLRP3 inflammasome, AQPs have been proposed to play a key role in several physiological processes needed in immune cells, such as migration and phagocytosis, to this purpose, AQP3 and AQP9 have been proposed as the most relevant AQPs isoforms in the immune system being able to regulate the migration of different immune cells (Rump and Adamzik 2018), AQP3 was reported to play a key role in the motility of T-cells and Macrophages (Hara-Chikuma et al. 2012), while AQP9 is involved in neutrophils cell migration (Rump and Adamzik 2018).
Interestingly, in leukocytes, AQP9 appears to be localized on cell edges, to facilitate lamellipodia formation, extension and stabilization, enabling these leukocytes to move towards chemoattractant (Loitto et al. 2002).

I.2.6. Aquaporin inhibitors

Several molecules have been proposed as inhibitors of various AQPs over the past few years, most of them being small molecules. Due to the similarity of AQPs and ion channels has caused a rising interest in ion channels modulator as possible AQPs inhibitors (Jeyaseelan et al. 2006), such as acetazolamides and anti-epileptic drugs (Tang and Yang 2016; Yang, Zhang, and Verkman 2008) but yet they still have to be proven as definitive AQP inhibitor (Abir-Awan et al. 2019).

Mercury chloride ($HgCl_2$) covalently binds to a pore-lining cysteine residue present in most AQPs, blocking the channel (Yang, Kim, and Verkman 2006). Despite its effectiveness, mercury and its compounds are toxic and unspecific, causing off-target effects (Castle 2005).

Heavy metal compounds have been also proposed as AQPs inhibitors, such as nickel chloride, which has been shown to inhibit AQP3 by linking to the extracellular loop residues Trp-128, Ser-152, His-241 (Zelenina et al. 2003). Copper sulfate has also been identified as an AQP3 inhibitor at a concentration of 100 μM sharing the same inhibiting mechanism with nickel chloride(Alejandra, Natalia, and Alicia 2018). Gold-based compounds which are used as AQP3 inhibitors such as AuPhen are capable of inhibiting glycerol permeability in erythrocytes with a concentration as low as 50 μM, and its effect can be reversed by the addition of the reducing agent 2-mercaptoethanol (Martins et al. 2012).

Other class of AQPs inhibitors are represented by the small molecule inhibitors, where phloretin is the most known AQP inhibitor, it acts as a non-specific aquaglyceroporin inhibitor inhibiting both AQP3 and AQP9 (Ishibashi et al. 1994; Tsukaguchi et al. 1998), despite its effectiveness it has been shown to exert off-target effects by inhibiting also the urea transporter, UT-A1, in kidney (Esteva-Font et al. 2013).

During the last years, several isoform-specific AQP inhibitory compounds have been identified by screening commercially available compound library using cell-based permeability assay (Sonntag et al. 2019).The compounds were subjected to *in silico* molecular docking, and compounds showing good docking properties were functionally tested on Chinese Hamster Ovary (CHO) cells expressing human AQP3 and AQP9.

The compound which showed the highest efficiency in blocking AQP3 is DFP00173, which affects the solute permeability of mouse and human AQP3 with an $IC_{50} \approx 0.1$ - 0.4 μM. This compound blocks AQP3 by interacting with the diclorophenyl group at the cytoplasmic opening of the pore and forming hydrogen bonds between the inhibitor urea linker and backbone carbonyls of loop B, as well as between the inhibitor nitro group and the asparagines of the NPA boxes (Sonntag et al. 2019).

The heterocyclic compound HTS13286 is a selective and potent inhibitory compound of AQP9 with an $IC_{50} \approx 2$ μM (Jelen et al. 2011). The linking site of HTS13286 was evaluated by site-directed mutagenesis and a cell water permeability assay of mutated AQP9 channels. Like phloretin, its interaction sites are in close proximity to histidine 82 and methionine 91 (Wacker, Aponte-Santamaria, et al. 2013). However, its poor water solubility limits its use *in vivo*, requiring optimization to improve hydrophilicity, pharmacodynamic and pharmacokinetic properties.

Recently a novel and potent inhibitory compound has been identified and characterized by our laboratory in collaboration with the Swedish company ApoGlyx, RG100204 (Florio et al. 2022; Mohammad et al. 2022). The compound is described as example 29 in the patent US20190127360 description of the synthetic route and the LC-MS and 1H NMR data.

Florio and coll. calculated the AQP9-mediated permeabilities to water ($IC_{50} \approx 5 \times 10^{-8}$ M) and glycerol ($IC_{50} \approx 5 \times 10^{-8}$ M) (Florio et al. 2022) in CHO overexpressing human AQP9.

Aquaporins might represent novel drug targets in sepsis, for instance, studies conducted on *AQP9*$^{-/-}$ mice showed higher survival rates and reduced inflammation when compared to *AQP9*$^{+/+}$ mice after LPS-induced endotoxic shock (Tesse et al. 2021). In the same study, the AQP9 inhibitor HTS13286 was also used for AQP9 gating in FaO cells, preventing the LPS-induced inflammation (Tesse et al. 2021).

In later studies, the novel AQP9 inhibitor RG100204, was shown to normalize the oxidative stress and improve the survival of mice subjected to cecal ligation and puncture as a polymicrobial model of sepsis (Mohammad et al. 2022).

Development of novel AQP inhibitors still faces significant challenges, mostly due to potential off-target and side effects, in fact, many inhibitors reported in the literature have shown inconsistent effects, often related to nonspecific interactions(Rump and Adamzik 2024).

I.3. MAIN AIMS OF THE WORK

The main aims of the doctoral work were the following ones:

1. To develop a novel polyclonal antibody targeting the rat epitope of AQP9, allowing the precise localization and expression analysis of AQP9 in human leukocytes, as well as to facilitate the identification of AQP9-positive cell populations in future flow cytometry analysis;
2. To evaluate AQP3 and AQP9 gene expression in PBMC and neutrophils from septic patients and healthy donors, assessing their relevance as prognostic and diagnostic biomarkers in sepsis. Such analysis would have been then used to develop a Machine Learning based prognostic and diagnostic algorithm;
3. To characterize AQP9 expression and subcellular localization in an inducible acute promyelocytic leukemia cell line (NB4) to explore its role in neutrophil cell function and its potential regulatory mechanisms, providing a novel cell model for studying AQP9 involvement in immune responses;
4. To investigate the precise function/s of AQP9 in human neutrophils using a promyelocytic cell line (NB4 cells) induced to maturation and challenged with stimuli such as LPS, fMLP, IL-6 and/or TNF- α.

CHAPTER 1

Expression and subcellular localization of Aquaporin-3 and Aquaporin-9 in human neutrophils and PBMC

II.1.1. ABSTRACT

Aquaporins (AQPs) are integral membrane proteins that allow the transport of water and small solute across cell membranes, playing pivotal roles in maintaining cellular homeostasis. Among these, Aquaporin-3 (AQP3) and Aquaporin-9 (AQP9) which are classified as peroxiporins by allowing the movement of hydrogen peroxide in addition to water and small solutes, are emerging as key modulators in immune responses and immune cells functions. This chapter investigates the expression and subcellular localization of AQP3 and AQP9 in human neutrophils and peripheral blood mononuclear cells (PBMC).

Through RT-qPCR we highlighted differential expression of both AQPs in neutrophils and PBMC, showing a strong expression of AQP9 in neutrophils which didn't exhibit any expression of AQP3, coherently with the immunofluorescence profiles, on the other hand, slightly higher expression of AQP9 when compared to the AQP3 levels were found in PBMC, notably, through immunofluorescence analysis, AQP9 was found expressed in both Monocytes and lymphocytes, while AQP3 was found to be expressed mostly in monocytes.

Furthermore, the development and validation of our custom designed anti-rAQP9 antibodies provides a robust tool for future research and potential clinical applications to screen for AQP9 expressing sub population of circulating cells. Our findings suggest that AQP3 and AQP9 may represent biomarkers and therapeutic targets in inflammatory disorders, since the marked expression of AQP9 in neutrophils suggests a key role in inflammatory processes, while AQP3 expression in monocytes might be related to the hydrogen peroxide entry, mediating the activation of the NLRP3 inflammasome. This work lays the foundation for exploring the broader implications of aquaporin regulation in immune cell functionality and systemic inflammation.

II.1.2. INTRODUCTION

II.1.2.1. Neutrophils: key players in innate immune response and inflammation

Immune defenses in vertebrates have evolved in two distinct subsystems: the innate immune system and the adaptive immune system.
The innate immune system is characterized by a wide range of unspecific host response, such as the barrier function given by the epithelia and the use of germ-line encoded receptors against pathogens (PRRs) (Clark and Kupper 2005).
A feature of the innate immune system is the ability to respond rapidly to the infection or the tissue disruption. (Janeway and Medzhitov 2002)
In contrast, the adaptive immune system is characterized by somatically re-arranged receptor genes to virtually recognize every antigen that could enter the host body, making this system slower in pathogen response but flexible enough to fight any pathogen that has evolved to evade the innate immune system. (Clark and Kupper 2005)
The circulating White Blood Cells (WBCs) part of the innate immune system includes dendritic cells, Monocytes, Macrophages, Granulocytes and Natural Killer T-cells.
The most abundant circulating white blood cells are the neutrophils (50%-70% of total White blood cells) (Gorgens et al. 2013), part of the Polymorphonucleated cells (PMNs) and represent the first defense line against the invading pathogens.
Production of neutrophils occurs in hematopoietic cords located in the bone marrow and derive from a common committed myeloid progenitor cell (Liew and Kubes 2019) (CCMP), the daily estimated production rate of these cells has been reported to be $2*10^{11}$ cells/die (Borregaard 2010).
The maturation of the common hematopoietic stem cell to the mature neutrophil, is a fine regulated multi-step process during which occurs the specific granules production (Borregaard 2010) and the segmentation of the nucleus in lobes (Veda 2011), typical of neutrophils.
Once neutrophils are fully mature they are retained, before being released in the blood stream, in the bone marrow for 4-6 days, evidences have estimated a total amount of $6*10^{11}$ neutrophils forming a bone marrow reserve (Athens 1963; Dancey et al. 1976).
Traditionally, neutrophils have been classified as short-lived immune cells, with a half-life of 6/8 hours in healthy conditions in humans (Basu et al. 2002; Galli, Borregaard, and Wynn 2011), nevertheless, during the last years, this definition have been challenged due to evidences showing a lifespan of 5.4 days in humans (Pillay et al. 2010).
The mechanism of cells senescence and turnover for neutrophils remains poorly defined (Liew and Kubes 2019), although is well known that dying neutrophils are cleared by macrophages in liver, spleen and bone marrow (Hong et al. 2012; Shi et al. 2001).
They, as key mediators in innate immunity, regulate acute and chronic inflammatory processes and thanks to their granules and secretory vesicles, which contain antimicrobial enzymes and matrix proteinases, these cells are able to invade the inflamed tissues and clear it from pathogens (Hager, Cowland, and Borregaard 2010).
Interestingly, all immune cells can utilize different transcription factors to determine the cell behavior, altering protein functions, dictating functions during the infections, neutrophils are an exception where no specific transcription factors have been identified in activated neutrophils, therefore, no subset of neutrophils have been reported (Burn et al. 2021).

On the other hand, neutrophils show phenotypical heterogeneity reflecting maturation or activation states like during pathogens interactions, where heterogeneity is needed from different signaling pathways without the need of any further differentiation (Deniset et al. 2017; Kamenyeva et al. 2015).

II.1.2.2. PBMC as a source of novel biomarkers

Peripheral blood mononucleated Cells (PBMC) are identified as any blood cells with a round nucleus as lymphocytes, monocytes, Natural Killer (NK) cells and dendritic cells (Kleiveland 2015).
In humans, PBMC population proportions can vary between individuals, although, lymphocytes are in the range of 70-90%, monocytes can vary between 10-20% and dendritic cells are rarer accounting only for 1-2% of the PBMC total.
In physiological conditions circulating PBMC appear as resting and/or naïve cells, in fact, in the absence of an ongoing immune response, the largest fraction of the isolated PBMC (T-Cells) are mainly present as naïve T-cells or Memory T-cells (Kleiveland 2015).
Although PBMC-derived biomarkers are easily found in blood, their evaluation remain a challenge caused by their minute quantity and high fragmentation (Chang et al. 2017).
Due to their plasticity and ability to differently adapt to a wide variety of insults, PBMC represent a novel circulating source of biomarkers for a wide variety of disorders especially inflammatory disorders and cancer (Bluth et al. 2008; Edwards et al. 2007), due to their different gene expressions and methylations profiles in these cells during malignancies like cancer, coronary artery diseases (CAD), type II diabetes and Alzheimer's disease (Chen et al. 2011; Hoekstra et al. 2010; Huang et al. 2010; Showe et al. 2009; Voellenkle et al. 2010).

II.1.3. AIM OF THE STUDY

The work object of this chapter aimed at isolating pure and viable PBMC and neutrophils from the peripheral blood of healthy individuals to assess the expression and localization of AQP3 and AQP9. This to lay down the basis for subsequent studies detailed in chapter two. In addition, we characterized the specificity and affinity of our custom designed polyclonal antibody which recognizes the rat epitope of AQP9 to further prove these antibodies as valuable tool to screen for AQP9-expressing cells populations by flow cytometry.

II.1.4. MATERIALS AND METHODS

II.1.4.1. Immunomagnetic isolation of neutrophils from human Peripheral Blood

Human peripheral blood was collected in Vacutainer™ K3-EDTA tubes (The Becton, Dickinson and Company Corporate, Franklin Lakes, NJ, USA), and processed within 30 minutes upon collection.
Human neutrophils were isolated using an immunomagnetic negative selection method, using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL technologies, Vancouver, BC, Canada) according to the following protocol.
Blood was transferred to 15 mL round bottom tube and 50 µL/mL of blood of Antibody cocktail were added and then equal amounts of magnetic beads were added to the blood and left to incubate for 5 minutes at Room Temperature (RT).
Later, blood sample was diluted with PBS + 2 mM EDTA up to 14 mL and left to incubate in the magnet for 5 minutes at RT.
The enriched Neutrophil suspension was carefully poured out in a new tube where magnetic beads were added using the same amount previously used and left to incubate in the magnet for 5 minutes at RT to remove any excess cells that may have been left from the previous separation.
The cell suspension was poured off in a new tube and left to incubate again in the magnet for 5 minutes at RT to remove any excess beads, finally the enriched cells were poured off, ready for the next steps.

II.1.4.2. Density gradient method isolation of PBMC from human peripheral blood

To isolate PBMC from healthy donor SepMate™ tubes were used by carefully pipetting density gradient medium, Lymphoprep™ (STEMCELL technologies, Vancouver, BC, Canada), through the central hole of SepMate™ tube insert.
Then, an aliquot of human peripheral blood was diluted with an equal amount of PBS+2%FBS (Gibco, Grand Island, USA), carefully pipetting the diluted blood on the side of the SepMate™ tube.
The sample was centrifuged at 1200xG for 10 minutes at RT in a swing rotor centrifuge with brakes ON.
After centrifugation the top layer, enriched of PBMC, was poured off in a new tube and washed two times with PBS pelleting cells each time centrifuging at 300xG for 5 minutes at RT.
After washing, cells were ready for the next steps.

II.1.4.3. Total RNA extraction from isolated cells

Total RNA was extracted from $1*10^6$ cells using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol.
Cells pellets were lysed by using 750 µL of TRIZOL (Thermo Fisher Scientific, Waltham, MA, USA), later, 200 µL of Chloroform was added and mixed by shaking for 15 seconds.

Subsequently, the mix was centrifuged at 12000xG for 15 minutes at 4°C using the Phasemaker™ Tubes (Thermo Fisher Scientific, Waltham, MA, USA), enhancing the aqueous phase collection.
To the aqueous phase were added 1.5 volumes of 100% ethanol and were filtered through the spin column by centrifugation at 8000xG for 15 seconds to allow total RNA to bind to the column.
After binding the RNA to the column, it was washed and then eluted with 30 µL of RNAse free water.

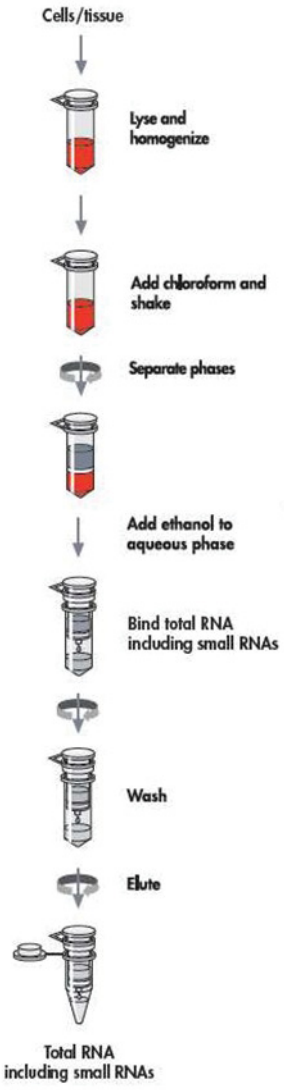


Figure 6 – **Flow chart of the protocol used to extract the total RNA from isolated neutrophils and PBMC.** Cells were lysed and the RNA enriched suspension was allowed to filter through the membrane of the spin-column by centrifugation and the total-RNA was then eluted by centrifugation with Nuclease-free water.

II.1.4.4. RT-qPCR analysis of AQP3 and AQP9 expression

To evaluate the transcriptional levels of expression of AQP3 and AQP9 in PBMC and neutrophils, we employed a semi-quantitative RT-qPCR analysis.
Firstly, 400 ng of total RNA extracted was retrotranscribed in cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA).

Reactions were prepared as described below:

RT-Master Mix	Volume (µL)
10x RT-Buffer	2.0
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2.0
Multiscribe Reverse Transcriptase	1.0
RNase Inhibitor	1.0
RNA + H_2O	Up to 20 µL of total Reaction
Total Volume of reaction	20 µL

Samples were placed in a in a MiniAmp Thermal Cycler (Applied Biosystems™, Waltham, MA, USA) with the following program:

Step	Temperature	Time
RNA denaturation	25°C	10 min
cDNA polymerization	37°C	120 min
Transcriptase degradation	85°C	5 min

After cDNA synthesis, each sample was quantified via Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and qPCR reactions for AQP3 (5' - GGAATAGTTTTT-GGGCTGTA -3' ; 5' - GGCTGTGCCTATGAACTGGT – 3'), AQP9 (5' - ATGTGGGAGCCCAG-TTCTTG - 3'; 5' - TACGGAGCTGGGTATGTTGC - 3') and β-Actin (5' - CTGTGGCATCCAC-GAAAC – 3'; 5' - CAGACAGCACTGTGTTGG -3') were carried out using the PowerUp™ SYBR™ Green Master Mix for qPCR (Applied Biosystems™, Waltham, MA, USA) as follows:

qPCR-Master mix	Volume (µL)
PowerUp™ SYBR™ Green Master Mix (2X)	5.0
Forward and reverse primers 8 µM (10X)	1.0
Nuclease-Free Water	3.0
cDNA template (50 ng)	1.0
Total volume of reaction	10.0

Samples were plated in duplicates and loaded in a StepOnePlus™ Real-Time PCR System (Applied Biosystems™, Waltham, MA, USA) with the following program:

Step	Temperature	Time	Cycles
UDG activation	50°C	2 min	1
Activation (Dual-Lock™ DNA poly-merase)	95°C	2 min	1
Denature	95°C	15 sec	40
Annealing	56°C	15 sec	
Extension	72°C	30 sec	

To prove the specificity of amplification, melting curves were performed after the last cycle using the program below:

Step	Ramp rate	Temperature	Time
1	1.6°C/sec	95°C	15 sec
2	1.6°C/sec	60°C	1 min
3	0.2°C/sec	95°C	15 sec

Relative expression for AQP3 and AQP9 was calculated using β-Actin as reference housekeeping gene according to the $2^{-\Delta Ct}$ method.

II.1.4.5. Immunofluorescence analysis of AQP3 and AQP9 on isolated cells

Immunofluorescence was performed to observe the localization of AQP3 and AQP9 in both PBMC and neutrophils isolated from healthy donor blood samples.
Cells were seeded on glass slides and incubated at 37°C for 7 minutes, then fixed with 4% paraformaldehyde for 20 minutes. Subsequently, cells were washed with PBS and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 20 minutes.
Cells were blocked in 3% BSA in PBS for 60 minutes at room temperature, then cells were incubated for 60 minutes with anti-AQP9 antibody (1:200) or anti-AQP3 antibody (1:200) diluted in PBS supplemented with 3% BSA. As a negative control, cells were incubated without the primary antibody.
Later, cells were washed with the same block solution and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000, #A-21206, Thermo Fisher Scientific) in PBS+3% BSA for 60 minutes at room temperature in the dark, subsequently slides were mounted, and nuclei were stained with Fluoroshield™ with DAPI (Merck).
Slides were observed with the Leica DMRXA fluorescent microscope and images were captured via Leica DFC450 C camera and processed using Leica Application Suite Advanced Fluorescence Software (LAS AF 3.1.0).

II.1.4.6. Cell lysis and Western blotting

Proteins from isolated PBMC and neutrophils were extracted by radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with Protease inhibitors (TB264086, Thermo Fisher Scientific) and then quantified via Pierce™ BCA protein assay kit (#23227, Thermo Fisher Scientific).
10 µg of proteins were denaturated by boiling for 5 minutes at 90°C, then protein samples were separated on a 4-12% polyacrylamide gel (Bolt™ Bis-Tris Plus mini protein gels, #NW04122BOX, Thermo Fisher Scientific) and electro-transferred to a polyvidene difluoride (PVDF) membrane (#IPVH00010, Merck) using a Mini Blot Module (#B1000, Thermo Fisher Scientific).
Membranes were blocked with 5% non-fat dry milk dissolved in Blotto (20 mM TRIZMA base, 150 mM NaCl, 1% Triton X-100) and subsequently incubated overnight at 4°C with our primary antibody rabbit anti-rAQP9 (1:1250).

Proteins were detected via incubation with goat anti-rabbit IgG-peroxidase (1:5000, #A0545, Merck) and subsequent reaction with ECL West Femto Plus Substrate (#ECL-2002, Società Italiana Chimici divisione scientifica) according to manufacturer instructions.
Membranes were then visualized using a Bio-Rad ChemiDoc.

II.1.4.7. Statistical analysis

Statistical analyses were carried out using GraphPad Prism software for Windows (version 9.0.0). Student's *t*-test was used to compare the different levels of expression between AQP3 and AQP9 in neutrophils and PBMC. In all cases a *p*-value less than 0.05 was considered significant.

II.1.5. RESULTS

II.1.5.1. Aquaporin-3 and Aquaporin-9 are differentially expressed in neutrophils and in PBMC

To assess Aquaporin-3 and Aquaporin-9 expression in neutrophils and PBMC we performed RT-qPCR analysis using β-Actin as reference gene.
From our analysis we evaluated the high expression of *AQP9* mRNA in neutrophils with a negligible, if not null, expression of *AQP3* mRNA (Fig. 7A), on the other hand in PBMC both *AQPs* are expressed at very low levels, with a higher expression of *AQP9* when compared to *AQP3* (Fig. 7B).

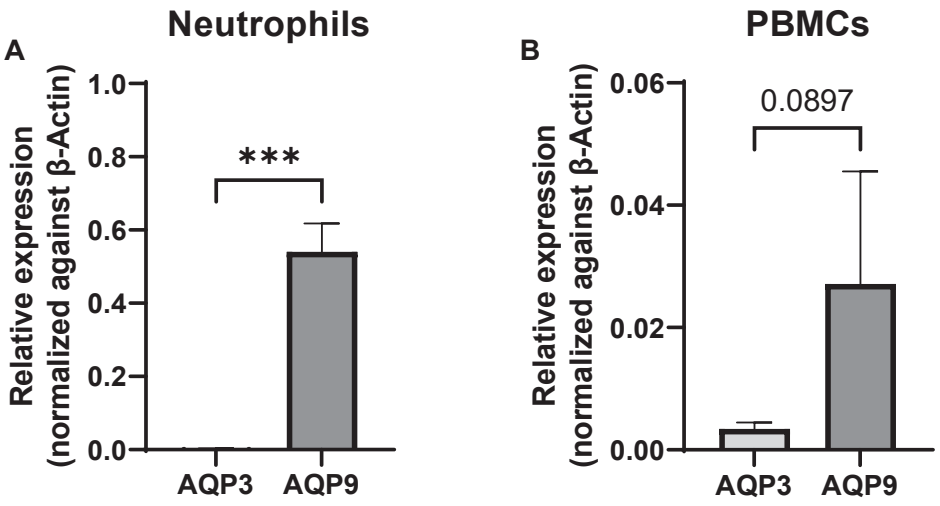


Figure 7 – **RT-qPCR analysis carried out on Immunomagnetic isolated neutrophils and density gradient method isolated PBMC.** (A) Relative expression levels of AQP3 and AQP9 in healthy donor neutrophils; *** $p < 0.001$; (B) Relative expression levels of AQP3 and AQP9 in healthy donors PBMC. Student's T-test was performed to compare levels of expression between AQP3 and AQP9

To further characterize the expression of both AQPs in neutrophils and PBMC we carried out also Western-Blot and Immunofluorescence analysis to evaluate protein expression and cellular localization.
Coherently with the RT-qPCR analysis, immunofluorescence on neutrophils showed a strong immunolabelling on the plasma membrane for AQP9 (Fig. 8).

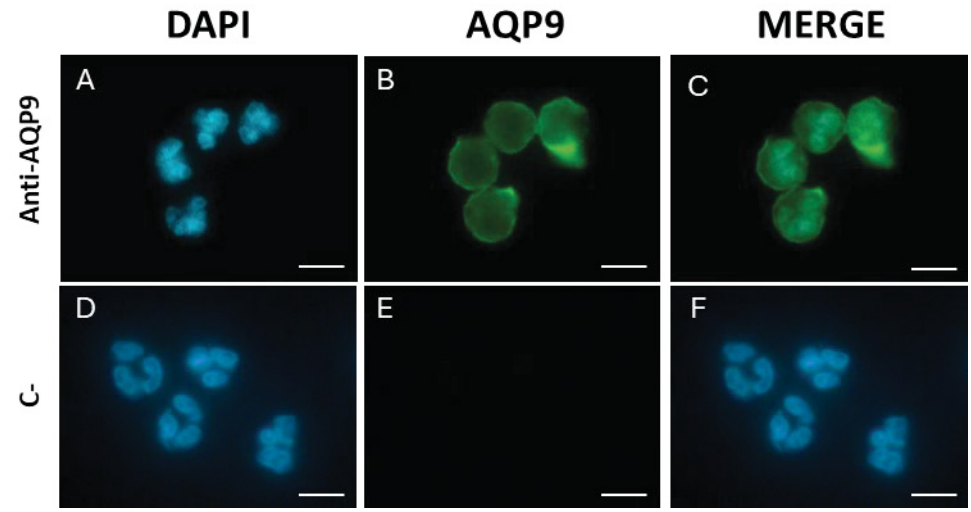


Figure 8 – **Immunofluorescence staining of AQP9 in neutrophil granulocytes.** (A and D) DAPI staining of cell nuclei; (B and E) Immunolabelling of AQP9 with a marked immunolabelling on the cell membrane in B and a negative signal, as expected, in Negative control (C-); (C and F) Merged images of DAPI-stained nuclei and immunolabeling of AQP9. Scale bar, 10 µm.

On the other hand, immunofluorescence staining on PBMC showed immunolabelling for both AQPs in both Lymphocytes and Monocytes. Immunolabelling for AQP3 was found brighter in monocytes when compared to lymphocytes, while AQP9 labelling was found in both lymphocytes and monocytes (Fig. 9).

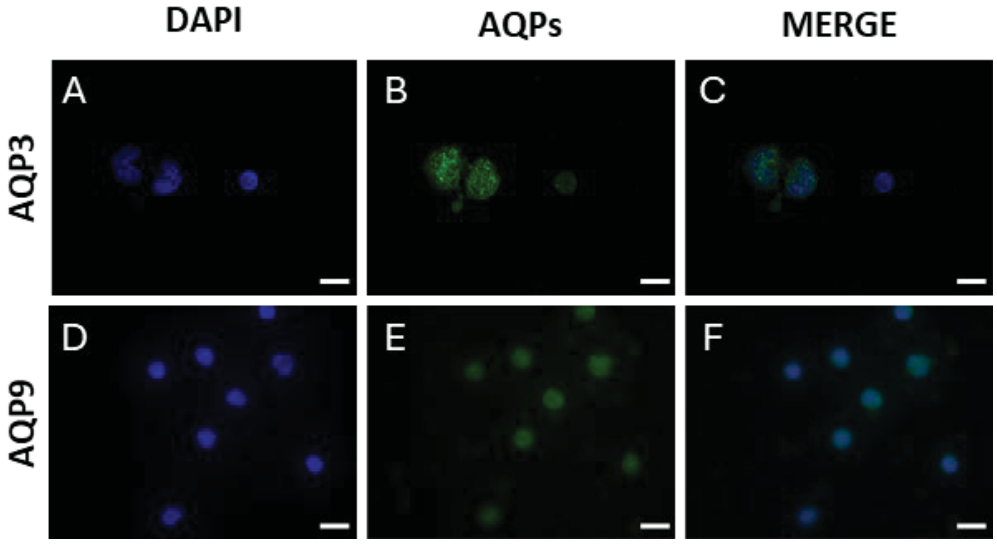


Figure 9 – **Immunostaining of Aquaporin-3 and Aquaporin-9 in PBMC.** PBMC were isolated from health subjects by SepMate methodology. (A and D) DAPI staining of PBMC cell nuclei; (B and E) Immunolabelling of AQP3 and AQP9 with a brighter immunolabelling for AQP3 in monocytes when compared to lymphocytes, while AQP9 could be seen in both monocytes and lymphocytes; (C and F) Merged images of DAPI-stained nuclei and immunolabeling of AQP3 and AQP9. Scale bar, 20 µm.

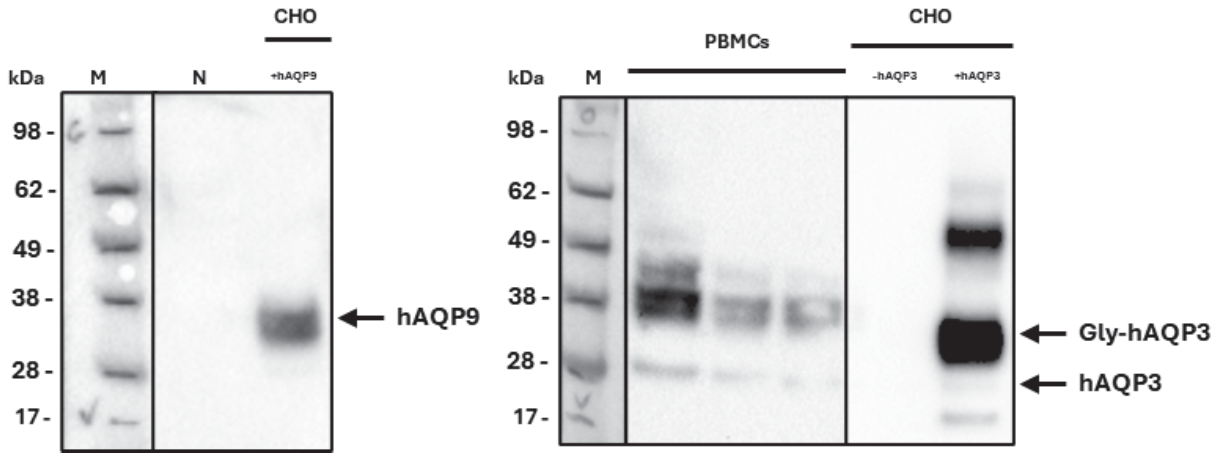


Figure 10 – **Western blotting analysis of AQP3 and AQP9 expression in isolated neutrophils and PBMC.** Commercially available antibodies for AQP3 and AQP9 were tested on cell lysates deriving from isolated neutrophils and PBMC. Abbreviations: M = Marker; N = Neutrophil lysate, CHO = Lysates of CHO cells transfected with the corresponding human Aquaporin; -hAQP3 = cells not induced to express hAQP3; +hAQP3 = cells induced to express hAQP3; +hAQP9 = cells induced to express hAQP9.

Same commercially available antibodies implied in immunofluorescence analysis were tested for Western-Blot, as pointed out by the Positive Controls, anti-AQP9 antibodies were not able to show any immunoreactivity when neutrophils cell lysates were probed, while PBMC cells lysates probed with anti-AQP3 antibodies detected a 28 kDa immunoreactive band representing the AQP3 monomer with a broader band between 30/35 kDa representing its glycosylated form (Fig. 10).
Due to the lack of reactivity by anti-AQP9 antibodies and the need for large amounts of specific and working antibodies to be implied in Flow Cytometry analysis, we decided to design an immunogenic peptide of AQP9 to commission the production of polyclonal antibodies in rabbit to the French company Covalab.

II.1.5.2. Design and test of our custom polyclonal Anti-rAQP9 Antibodies.

For the immunization process, rat-AQP9 epitopes were considered and amongst the three different immunogenic peptides that could be produced starting from the peptide sequence, the C-Terminus region appeared as the most immunogenic which could be synthesized and then used to immunize rabbits (Table 1).

Table 1 – **Peptide sequences analyzed for immunogenicity**.

AQP9 Region	Peptide Sequence	Immunogenicity
C-Terminus	C-DPDMKAEPSENNLEKHELSVIM-COOH	High
Loop C	C-VGENATAFIFATY-NH_2	None
N-Terminus	C-SEKDGAKKSLMQRLALKS-NH_2	Low

After the immunization and the rabbit sera affinity purification, we tested our custom antibodies on Western Blotting analysis by using CHO cells transfected with the human construct of AQP9 available in our laboratory.

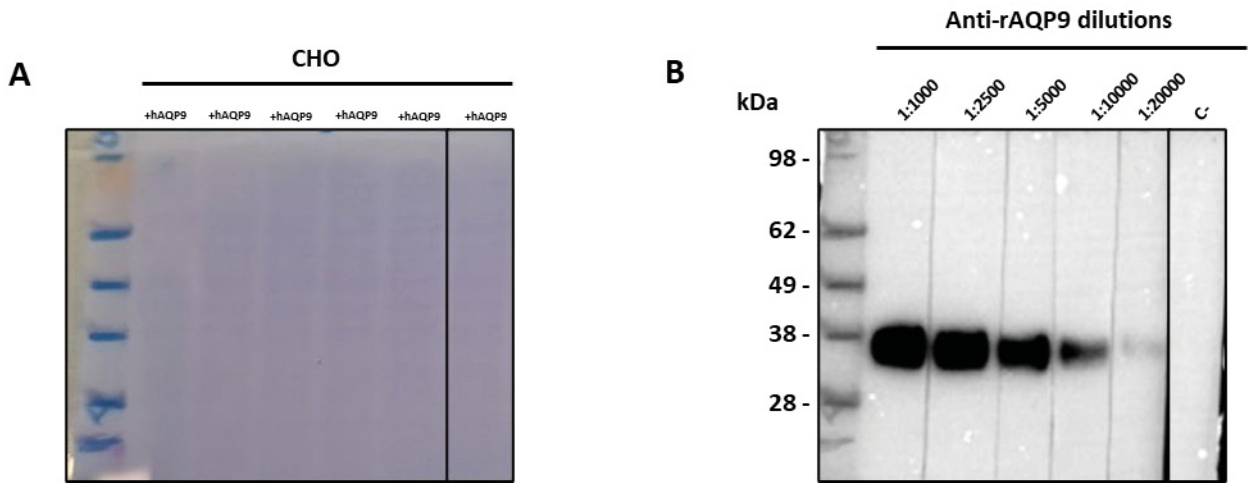


Figure 11 – **Western blotting analysis of committed anti-rAQP9 polyclonal antibody.** (A) Coomassie blue stained PVDF membrane, 1 µg of cell lysates were loaded on a polyacrylamide gel and then electro-transferred on a PVDF membrane. (B) Immunoreactive bands relative to hAQP9 incubated with serial dilutions of the custom polyclonal antibody.

Our custom polyclonal antibody showed strong and clear immunoreactivity on lysates deriving from cells overexpressing AQP9 under the control of the TET inducible promotor (Sonntag et al. 2019), with no unspecific bands with a broad range of dilutions from 1000 to 20.000 folds (Fig. 11).
Further evaluations are still ongoing to prove the same specificity and reactivity on human AQP9 extracted from circulating immune cells.

II.1.6. DISCUSSION

The work object of this chapter was aimed at assessing the validity of commercially available antibodies as tools to probe the expression of AQP9 and AQP3 in a wide range of tissues and organisms, since the lack of specificity from antibodies directed against AQPs is still a debated discussion (da Silva et al. 2022).
By RT-qPCR analysis and immunofluorescence, we described that AQP3 and AQP9 are differentially expressed in both immune cell types, AQP9 is mainly expressed in neutrophils with a lower expression in both monocytes and lymphocytes, while AQP3 appears mainly expressed in Monocytes suggesting different roles for the two Aquaporins in the two cell types.
The involvement of the two AQPs in different physiological and pathophysiological pathways might be investigated through the usage of commercially available inhibitory compounds, making them possible pharmacological targets.
The differential expression and the time-dependent expression of AQP3 and AQP9, which will be a topic in the third chapter, during the Neutrophil maturation, might be exploited as feasible markers to be implied in Flow Cytometry (FC) analysis to screen for Neutrophil populations in blood samples.
Due to the nature of the AQPs most of the commercially available antibodies lack of specificity and cannot be implied in FC analysis, on this topic, we designed and produced our custom polyclonal antibody against rat-AQP9 which showed strong and specific immunoreactivity in both immunofluorescence and western-blotting analysis, further evaluation are still needed and are ongoing to prove their specificity also on human samples, to pave the way for their use in Flow Cytometry analysis.

II.1.7. CONCLUSION

In conclusion, we found strong expression of AQP9 in neutrophils and AQP3 in monocytes. Previous work from our laboratory outlined AQP3 as the possible pathway through which hydrogen peroxide, a molecule contributing to the activation of the NLRP3 inflammasome, flows within monocytes. The custom anti-AQP9 antibodies produced during the PhD work show good requisites to be used to investigate the relevance of AQP9 as biomarker and drug target in sepsis. AQP9 as possible biomarker in sepsis is object of the work described in chapter 2.

CHAPTER 2

AQP3 and AQP9 novel promising diagnostic and predictive prognostic biomarkers by Machine Learning-driven approaches

II.2.1. ABSTRACT

Aquaporins are reported to play critical roles in inflammatory disorders such as those underlying sepsis. However, their potential as biomarkers in sepsis is currently poorly investigated. The work covered in this chapter consisted of a pilot study conducted on 47 subjects among healthy donors and septic patients admitted to the ICU of the "*Guido Baccelli*" Clinic at the Department of Precision and Regenerative Medicine and Ionian Area (DIMEPRE-J) of the University of Bari. The work was done in collaboration with Prof.s Antonio Solimando, Massimo Bilancia, Vanessa Desantis and Angelo Vacca. Bedside data, hematological parameters and main septic scores (SOFA, qSOFA and NEWS2) from each patient together with the data obtained from the healthy subjects were recorded and stored in an anonymized database and then merged with the expression levels of AQP3 and AQP9 measured by RT-PCR in neutrophils and PBMCs isolated from blood sample aliquots of the same patients. All data were merged using a specific prognostic and diagnostic ML-driven algorithm specifically set up for the study in question. Increased expression of AQP3 (about 30%; $p < 0.01$) is seen in the PBMCs of septic patients compared to the healthy donors while no increase was found regarding neutrophil AQP9 when comparing septic patients with healthy subjects. Interestingly, the expression levels of AQP9 and AQP3 in septic patients showed significant correlations with the NEWS2 septic score, serum lactate levels and circulating Procalcitonin levels, respectively.
Altogether these results were used to train our prognostic and diagnostic ML-driven algorithm based on random forest classification, highlighting promising results both for prognostic and diagnostic applications of the two AQPs as markers in the diagnosis of and septic mortality prognosis.

II.2.2. INTRODUCTION

II.2.2.1. Sepsis diagnosis and management

For years sepsis has been described as an overabundant inflammatory response to infection, recognized by the presence of the systemic inflammatory response syndrome (SIRS), which is defined as two or more abnormalities of temperature, heart rate, respiratory rate or white-cell count (You et al. 2025).

Over the decades, numerous medical reports have proposed novel definitions for sepsis as well as novel ones for septicemia, toxemia, bacteremia and endotoxemia (Singer et al. 2016).

To provide a clear definition of Sepsis the international academic community revised the definition and diagnosis of sepsis thrice, defining it as "*a life-threatening organ dysfunction caused by a host's dysfunctional response to infection*" (David and Brunkhorst 2017).

This new definition emphasizes the mechanism and severity of infection-induced organ dysfunction requiring a timely diagnosis and clinical treatment.

The novel diagnostic criteria differ between intensive care unit (ICU) patients and non-ICU patients. In case of ICU patients with infection or suspected infection, the diagnosis of sepsis is accepted when the Sequential Organ Failure Assessment (SOFA) score is 2 points, while, for non-ICU patients the diagnosis of sepsis is accepted whenever there are two quick Sequential Organ Failure Assessment (qSOFA) scores (systolic blood pressure less than 100 mm Hg, respiratory rate loss greater than 22 times/min, a change in consciousness) or more than two positive diagnosis pointing out to sepsis (Seymour et al. 2016; Shankar-Hari et al. 2016).

With the new definition of sepsis, also the definition of septic shock, its most severe complication, has been redefined to include all the circulatory and cellular metabolic disorders caused by sepsis, which lead to a higher risk of death than the one registered from sepsis only (Peach 2017; Singer et al. 2016).

Septic shock patients can be clinically identified by their need of vasopressors to maintain a mean arterial pressure of 65 mmHg or higher, and with a serum lactate level above 2 mmol/L without hypovolemia (Singer et al. 2016).

It has been reported that the current qSOFA score might be too sensitive with a low specificity leading to a overdiagnosis of sepsis, moreover its predictive value has been suggested to be lower than other scoring systems (Churpek et al. 2017; Simpson 2016).

Despite the advancements in the Medical field, sepsis still remains a major public health concern both for patients and the national health care system, with its length of stay in the ICUs often extending between 5 and 20 days (Arefian et al. 2017; Prkno et al. 2013; Tiru et al. 2015).

Altogether, these considerations show the burden imposed on patients, families and the National health care system, demonstrating the need for an early diagnosis and personalized treatment for sepsis.

II.2.2.2. Prognostic and diagnostic biomarkers implied in sepsis management

Due to the high heterogeneity of symptoms evaluated in septic patients and the need for a timely diagnosis, during the last years a multitude of biomarkers have been proposed, which could be implemented to screen for septic patients with a diagnostic and prognostic value.

Most of the biomarkers proposed are related to the immune system activation, which is the major player during septic inflammation, in fact C-Reactive Protein (CRP) and Procalcitonin (PCT) are the most widely used and studied biomarkers (Pierrakos et al. 2020).

Serum levels increase transiently for both proteins during sepsis and although PCT is considered a superior biomarker when compared to CRP (Luzzani et al. 2003; Simon et al. 2008), its low specificity has been demonstrated due to its serum levels increase also in other conditions not related to sepsis (Park et al. 2014). It has been proposed that PCT, like CRP, might be more useful in ruling out sepsis instead of diagnosing it (Chan et al. 2002; Hattori et al. 2014; Kono et al. 1999).

Another class of compounds that have been proposed as possible biomarkers are cytokines like TNF-α and IL-10 which were found to be increased during the first 24 hours after the admission of the patient (Pinsky et al. 1993), unfortunately blood cytokine concentration time course is still not clear during sepsis time course (Pinsky et al. 1993; Wu et al. 2009), making its interpretation not reliable for diagnostic purposes.

Cell surface molecules have been also proposed as diagnostic markers, such as sTREM-1 and CD64. sTREM-1 is a soluble form of TREM-1 (Triggering receptor expressed on myeloid cells) which is a cell surface molecule expressed mainly in monocytes and neutrophils which plays a role in the inflammatory and cytotoxic response to sepsis synergistically activating the Toll-like receptors and the increase of production of pro-inflammatory cytokines (Bleharski et al. 2003), several studies have demonstrated that serum levels of sTREM-1 are a useful diagnostic biomarker for sepsis (Gomez-Pina et al. 2007) (Table 2).

CD64, a high affinity Fcγ receptor I, is present at low levels on resting neutrophils and its expression is up-regulated in the early phases of activation of the immune response, due to its changes in expression it has been proposed as sepsis biomarker in several studies (Ye et al. 2019; Yeh et al. 2019; Yin et al. 2020).

Table 2 – **Current diagnostic biomarkers of sepsis used in clinical medicine.** AUC = Area under the ROC curve. From (Kim and Choi 2020)

Category	Biomarker	Ability to diagnose	AUC	Ref
Soluble receptors	Presepsin	Sepsis	0.954	(Lu et al. 2018)
	sTREM-1	SIRS or Sepsis	0.78	(Aksaray et al. 2016)
		Septic shock	0.955	(Brenner et al. 2017)
	suPAR	Sepsis	0.99	(Khater et al. 2016)
Membrane receptors	CD64	Sepsis	0.879	(Yin et al. 2020)
DAMP	Calprotectin	Sepsis	0.607	(Larsson et al. 2020)
CC chemokine ligand 2	MCP-1	Septic shock	0.716	(Tian et al. 2019)
miRNA	miR-125a	Sepsis	0.749	(Zhao et al. 2020)
	miR-125b	Sepsis	0.839	
Non-coding RNAs	Lnc-MALAT1	Sepsis	0.91	(He, Zhang, and Huang 2019)
		Septic Shock	0.83	
	Lnc-MEG3	Sepsis	0.88	(Na et al. 2020)

It should be noted that the same diagnostic biomarkers have been also proven to be of prognostic relevance in septic patients, with the addition of molecules which are mainly involved during organ dysfunction caused by sepsis (Kim and Choi 2020). (Table 3)

Table 3 – **Current prognostic biomarkers of sepsis used in clinical medicine.** AUC = Area under the ROC curve. From (Kim and Choi 2020)

Category	Biomarker	Ability to predict	AUC	Ref
Soluble receptors	suPAR	28-day mortality	0.77	(Casagranda et al. 2015)
CC chemokine ligand 2	MCP-1	30-day mortality	0.64	(Barre et al. 2018)
Acute phase protein	PTX-3	28-day mortality	0.82	(Kim et al. 2017)
	MR-proADM	28-day mortality	0.79	(Andaluz-Ojeda et al. 2017)
	ADM	30-day mortality	0.827	(Kim et al. 2019)
Angiogenic growth factor	Angiopoietin	28-day mortality	0.736	(Seol et al. 2020)
miRNA	miR-223	28-day mortality	0.748	(Wang et al. 2012)
	miR-15a		0.610	
	miR-16		0.76	
	miR-122		0.744	
	miR-193b		0.79	
	miR-483-5p		0.657	
Non-coding RNAs	Lnc-NEAT1	28-day mortality	0.726	(He, Zhang, and Huang 2019)
	Lnc-MALAT1	Mortality	0.886	(Chen et al. 2020)
	Lnc-MEG3	28-day mortality	0.704	(Na et al. 2020)

II.2.2.3. Artificial intelligence applications to sepsis clinical management

Since 2012, thanks to the evolution of computer science, computing capabilities and newer Machine-Learning Algorithms, Artificial Intelligence (AI) expanded throughout every day-to-day life aspect, approaching also Clinical healthcare.

Basic Machine Learning uses Algorithms to learn from the data, using the learning results to make decisions and predictions about upcoming events (Wu et al. 2021).

Machine learning appeared during the early stages of Artificial Intelligence development, and unlike traditional hard-coded software it uses numerous amounts of data to learn how to perceive a specific task.

The first applications of a traditional ML algorithm in sepsis management were extremely difficult, until the rise of a newer Algorithm, known as Deep Learning (Wu et al. 2021).

The original and first system implied in sepsis prediction relied mainly on empirical clinical decision rules (CDR) which usually implies bedside vital signs such as heart rate, respiratory rate and blood pressure, these data were then classified by a SVM classifier, generating a model which can predict sepsis with an accuracy of 83%, and an AUROC (Area Under Receiving Operating Characteristics) of 0.781 (Mohammed et al. 2021), screening all the hospitalized patients, passing automatically the results to the clinicians without the human supervision.

Unfortunately, as a trade-off, the main disadvantage of CDR is given by its low applicability to a population different from the one used to train it, leading to performances issues.
With the progress of Deep-Learning, more studies have been conducted, comparing its performance to three machine learning algorithms (Random Forest, Cox Regression and penalized logistic regression) and three scoring screening tools (SIRS, qSOFA and NEWS), strikingly, the deep-learning method and the Random Forest Algorithm outperformed the other screening tools when tested using the same datasets (Bedoya et al. 2020).
On the other hand, the Random Forest algorithm approach, has been proven to be the most accurate and specific in the early detection of septic shock, with an AUROC of 0.9483 (Misra et al. 2021).
Due to the complexity of AI systems, it is difficult to understand accurately how the AI makes decisions, leaving the human clinicians without any control on the AI-based decision (Habli, Lawton, and Porter 2020), moreover when a machine based system interprets data it cannot reason the data like a human clinician which might decide to omit some data due to a sampling, analysis or recording error.
Nowadays the major limitations on the application are the training and validation process, most of the studies rely on the same patients cohort for both processes, leading to an inadequate validation on the heterogeneity of the population which is hospitalized (Schinkel et al. 2019), moreover, most of the AI applications will find difficulties in being integrated in the current clinical workflow, since most healthcare organizations may not have the infrastructure needed to successfully integrate these newer systems.

II.2.2.4. Therapeutical strategies in sepsis fighting

Today, the gold-standard therapy for sepsis mainly consists in eliminating any invasive surgical procedures and timely administration of targeted antibiotics. Moreover, in case of organ failure, additional intensive care measures are implied for organ support, like vasopressor administration, mechanical ventilation and renal replacement therapy (Jarczak, Kluge, and Nierhaus 2021).
Since the timely intervention is crucial in managing sepsis, based on latest guidelines (2016), the so called “Hour-1-Bundle” consist of 5 clinical interventions: blood cultures, administration of broad-spectrum antibiotics (which should be administered as soon as possible), administration of IV fluid, administration of vasopressors and measurement of lactate levels.
The key for a successful therapy is the elimination of the underlying infectious agent and infection source, which cause the continuous immune system imbalance, so as soon as the pathogen has been identified the anti-microbial therapy has to be narrowed down with specific antibiotics (Kumar 2016), increasing the probability of survival.
Another key element in the sepsis management is the hemodynamic management to keep blood pressure stable, which is needed due to the external fluid loss always present in sepsis cases as well as vasodilatation and capillary leakage (Kumar 2016; Vincent 2022).
To reduce the abnormal activation of the immune system, purification of blood from PAMPS and DAMPS by blood purification methods has been theorized to be a reasonable approach (Jarczak, Kluge, and Nierhaus 2021). Extracorporeal blood purification derive from Renal Replacement Therapy (RRT) and examples are high volume hemofiltration (HVHF) and plasmapheresis for the absorption of endotoxins and cytokines, unfortunately, although they have been shown to be able to com-

pletely remove inflammatory mediators and bacterial toxins, there is still lack of evidences showing their efficacy in sepsis treatment (Honore et al. 2012).

Despite the progress in knowledge of the complex balance of the immune system, the gold-standard for sepsis treatment still remains the timely administration of anti-microbial drugs and the hemodynamic stabilization, since the previous approach of anti-inflammatory drugs therapies has been proven unsuccessful during the last years, a shift in the investigation must take place towards modulating and re-balancing the altered immune response during sepsis and septic shock (Jarczak, Kluge, and Nierhaus 2021; Vincent 2022).

II.2.3. AIM OF THE STUDY

The main aim of the work object of this chapter was to run a pilot study with blood samples of septic patients (vs. healthy donors) to evaluate the possible relevance of leukocyte AQP3 and AQP9 as diagnostic and prognostic biomarkers of sepsis.

II.2.4. MATERIALS AND METHODS

II.2.4.1. Recruitment criteria of septic patient cohort and clinical data collection.

Forty-seven septic patients were recruited from hospitalized patients in the "*Dipartimento di Medicina di Precisione e Rigenerativa e Area Jonica"* (DiMePRe-J)*, Azienda Ospedaliero Universitaria Consorziale Policlinico di Bari*, after informed consent and approval of the Local Ethics Committee.

For the Control group, 25 healthy donors were selected.
The inclusion criteria for patients were defined as follows:

- Age included between 18 and 85 years of age.
- SOFA score higher or equal than 2 to prove the organ disfunction.

The following groups of patients were excluded from the analysis:

- Patients receiving antibiotic therapy for more than 72 hours
- Patients with ongoing neoplasms
- Pregnant women
- Patients receiving immunosuppressing therapy
- Patients with heart failure (NYHA)
- Liver failure classified as B or C according to the Child-Pugh classification
- Terminally ill patients with a life expectancy less than 48 hours
- Patients characterized by a medical history of inflammatory or hematic disease.

II.2.4.2. Total RNA extraction from whole peripheral blood collected from septic patients

Total RNA was extracted from isolated cells using the phenol/chloroform method by using TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer protocol.
Cells pellets were lysed by adding 1 mL of TRIZOL (Thermo Fisher Scientific, Waltham, MA, USA), later, 200 µL of Chloroform was added and mixed by shaking for 15 seconds.
Subsequently, the mix was centrifuged at 12000xG for 15 minutes at 4°C using the Phasemaker™ Tubes (Thermo Fisher Scientific, Waltham, MA, USA), enhancing the aqueous phase collection.
Once collected the aqueous phase, it was mixed with 500 µL of isopropanol and incubated for 10 minutes at room temperature (RT), later it was centrifuged for 10 minutes at 12000xG at 4°C to precipitate the total RNA.
The supernatant was discarded, and the pellet was resuspended in 1mL of 75% ethanol then it was centrifuged for 5 minutes at 7500xG at 4°C.
The supernatant was again discarded, and the pellet was air dried for 10 minutes at RT, later the pellet was resuspended in RNAse free H_2O and quantified by using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for downstream applications.

II.2.4.3. RT-PCR of Aquaporin-3 and Aquaporin-9 in septic patient peripheral blood samples

To evaluate the transcriptional levels of expression of AQP3 and AQP9 in PBMC and neutrophils isolated from healthy donors and septic patients, we employed a semi-quantitative RT-PCR analysis.

Firstly, 800 ng of total RNA extracted was retrotranscribed in cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA).
Reactions were prepared as described below:

RT-Master Mix	Volume (µL)
10x RT-Buffer	2.0
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2.0
Multiscribe Reverse Transcriptase	1.0
RNase Inhibitor	1.0
RNA + H_2O	Up to 20 µL of total Reaction
Total Volume of reaction	20 µL

Samples were placed in a in a MiniAmp Thermal Cycler (Applied Biosystems™, Waltham, MA, USA) with the following program:

Step	Temperature	Time
RNA denaturation	25°C	10 min
cDNA polymerization	37°C	120 min
Transcriptase degradation	85°C	5 min

Once the cDNA synthesis was finished, the PCRs reactions were prepared to amplify the AQP3 and AQP9 cDNA, β-Actin cDNA was also amplified as reference gene and internal control.
Reactions were prepared accordingly to the following composition by using the Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA):

PCR-Master Mix	Volume (µL)
10X Taq Buffer	2.5
dNTP Mix, 2 mM each	0.5
Forward primer	0.1-1.0 µM
Reverse primer	0.1-1.0 µM
25 mM $MgCl_2$	2.0
Taq DNA Polymerase	0.25
Template DNA	4.5
Nuclease-free H_2O	Up to 25 µL
Total volume of reaction	25 µL

Samples were then placed in a MiniAmp Thermal Cycler (Applied Biosystems™, Waltham, MA, USA), setting the following program:

Step	Temperature	Time	Cycle
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	1 min	30
Annealing	59 °C	30 sec	
Polymerization	72 °C	1 min	
Final polymerization	72 °C	10 min	1

Each sample was run in triplicate for the subsequent analysis.

II.2.4.4. Semi-quantitative analysis of expression of AQP3 and AQP9

PCR amplification products were mixed with 6X DNA Gel Loading Dye (Thermo Fisher Scientific, Waltham, MA, USA) and loaded in a 1.5% Agarose gel, stained with *GelGreen® Nucleic Acid Gel Stain* (Merck Millipore, Burlington, MA, USA).
Once the gel electrophoresis was finished, the gel was visualized through a PrepOne™ Sapphire Blue LED Illuminator (Embi Tec, San Diego, CA, USA) and photographed.
To proceed with the analysis of expression, the image was imported in ImageJ and transformed in an 8-bit Grayscale image where optical density for AQP3, AQP9 and β-Actin bands were recorded on an Excel Spreadsheet.
To calculate the levels of expression of AQP3 and AQP9, both optical densities were normalized against the optical density registered from the β-Actin deriving from the same sample, averaging the levels of expressions calculated for each replicate.

II.2.4.5. Cytotoxicity analysis of AQP3 inhibitor.

JURKAT cells were seeded into a 96-well plate at a density of 10^4 cells/well in RPMI1640 (Biowest) supplemented with 10% FBS (Euroclone) and 1% Penicillin/Streptomicin (Euroclone) and were incubated for 24h with or without dilutions (1.25 μM – 10 μM) of the investigated inhibitor.
The MTT colorimetric assay is based on the cleavage of yellow tetrazolium salt to purple formazan crystal by metabolic active cells. The MTT solution was added to the cells and incubated at 37°C for 4h. Then the formazan crystals were solubilized in DMSO, and the optical density was measured at 540 nm by NanoDrop 1000 (Thermo Fisher Scientific Inc.).
The absorbance was then compared to untreated samples and represented as the percentage of living cells.

II.2.4.6. Measurement of H_2O_2 cell permeability.

Jurkat cells were seeded on a coverslip at 200.000 cells/coverslip density and incubated in complete medium (RPMI1640+10%FBS+ 1% PEN/STREP) for 1h at 37°C in 5% CO_2.
Then the cells were loaded with 10 μM H_2-DCFDA (Invitrogen) for 30 min at 37°C in 5% CO_2 and mounted in a closed chamber (Warner instrument) on the stage of a Zeiss Axiovert 200 inverted microscope, using a 40X epifluorescence objective.
The fluorescence signal was acquired in 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 100s, followed by 300 μM H_2O_2.
Fluorescence was recorded with excitation at 495 nm, with 10 nm bandwidth, and emission was collected with a 510/10nm bandpass filter.
Data were recorded and analysed using the Metafluor software (Molecular Devices), connected to a CCD camera (Cool Snap TM EZ, Photometrics).

II.2.4.7. Immunofluorescence analysis of AQP3 in JURKAT cells

Immunofluorescence was performed to observe the localization of AQP3 in basal JURKAT cells
Cells were seeded on glass slides and incubated at 37°C for 7 minutes, then fixed with 4% paraformaldehyde for 20 minutes. Subsequently, cells were washed with PBS and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 20 minutes.
Cells were blocked in 3% BSA in PBS for 60 minutes at room temperature, then cells were incubated for 60 minutes with anti-AQP3 antibody (1:200) diluted in PBS supplemented with 3% BSA. As a negative control, cells were incubated without the primary antibody.
Later, cells were washed with the same block solution and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000, #A-21206, Thermo Fisher Scientific) in PBS+3% BSA for 60 minutes at room temperature in the dark, subsequently slides were mounted and nuclei were stained with Fluoroshield^{TM} with DAPI (Merck).
Slides were observed with the Leica DMRXA fluorescent microscope and images were captured via Leica DFC450 C camera and processed using Leica Application Suite Advanced Fluorescence Software (LAS AF 3.1.0).

II.2.4.8. Statistical analysis correlating AQPs expression and clinical parameters

Anonymized clinical parameters datasets and Aquaporins expression levels were loaded in R Statistical Software (R Core Team (2021), https://www.R-project.org/), where datasets were aligned according to an anonymized code representing patients, all was done by using the R library “tydiverse”.
All comparisons between the 47 septic patients and the 25 healthy donors were performed by statistical Student’s t-test, considering a $p \leq 0.05$ statistically significant.
The correlation analysis between couples of continuous variables was performed by calculating the correlation coefficient by the Pearson correlation test.
Scripting, modelling and testing the algorithm was done through Python programming language and ensemble algorithms.
All data were then exported to GraphPad Prism version 9.0.0 for Windows (GraphPad Software, Boston, MA, USA) for plotting purposes.

II.2.5. RESULTS

II.2.5.1. Demographic and clinical characteristics of patients

The tables below present the demographic, hematological, and bedside data collected from septic patients recruited in our pilot study. Initially, we gathered clinical follow-up and outcome data for each patient, providing foundation for subsequent analyses. From our cohort of patients 22% of patients didn't survive the infection, while 78% of patients survived.

Table 4 – **Demographic summary table of patients used for the analysis.**

Variable	Male	Female
Age (±SD)	65 ± 17	73 ± 11
Length of hospital stay (±SD)	30 ± 19	25 ± 17
Death	6	5
Survive	19	17

All patients were subjected to a peripheral blood withdrawal to evaluate hematological parameters such as White Blood Cells (WBC) count and main inflammatory markers levels like C-reactive protein (CRP), Procalcitonin (PCT) and fibrinogen.

Table 5 – **Hematological parameters collected from recruited patients**

Variable	Average values	
	Male	**Female**
WBC n° (±SD)	11727 ± 19296	14774 ± 22722
Neutrophils n° (±SD)	5801 ± 5268	7661 ± 5204
Lymphocytes (±SD)	1736 ± 2286	6273 ± 23012
NLR (±SD)	7 ± 12	27 ± 91
Monocytes n° (±SD)	398 ± 238	658 ± 765
Eosinophils (±SD)	207 ± 502	115 ± 173
Basophils (±SD)	34 ± 45	44 ± 74
CRP (±SD)	66 ± 56	92 ± 55
PCT (±SD)	2 ± 4	13 ± 28
Fibrinogen (±SD)	318 ± 129	403 ± 150

To better stratify patients, bedside data (currently used to evaluate the risk of sepsis) were collected to calculate septic scores like SOFA (Sequential Organ Failure Assessment) and NEWS2 (National Early Warning Score-2).

Table 6 -**Bedside data and septic scores collected by patients divided by gender**

Variable	Average values	
	Male	**Female**
SBP (±SD)	114 ± 16	118 ± 20
DBP	69 ± 12	65 ± 10
MAP	86 ± 8	86 ± 12
Respiratory Frequency	17 ± 4	17 ± 3
Temperature	37 ± 1	37 ± 0.5
GCS	15 ± 0.6	14 ± 2.9
SOFA	4 ± 3	3 ± 2
NEWS2	4 ± 2	6 ± 2

All the data collected were not significantly different between genders, highlighting that the stratification of patients based on gender is not required.

II.2.5.2. Aquaporin-3 is up-regulated in septic patients compared to healthy donors

To test the hypothesis whether AQP3 and AQP9 could be used as easily analyzable prognostic and diagnostic biomarkers for septic conditions we carried out relative-expression analysis by RT-PCR on total RNA extracted from healthy donors and septic patients PBMC and neutrophils.

The RT-PCR analysis highlighted no differences in gene expression of *AQP3* and *AQP9* mRNA between gender groups, showing no effect of gender on *AQPs* expression in both groups (Fig. 12A and 12B).

Nevertheless, we evaluated a 30% increase of *AQP3* expression in septic patients PBMC when compared to the healthy donors ($p < 0.01$), in contrast, no significant differences were evaluated in *AQP9* expressions levels (Fig. 12C).

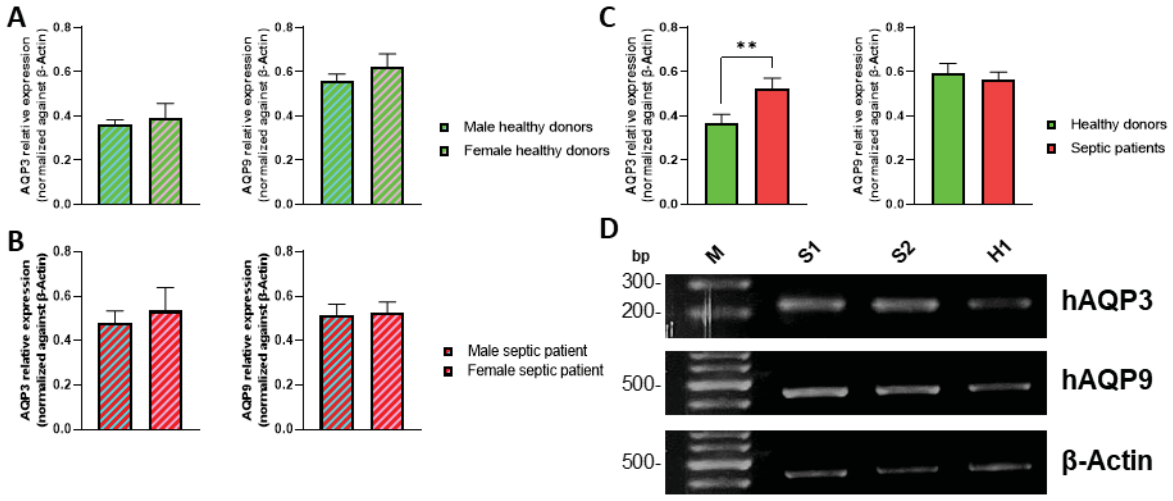


Figure 12 – **Expression levels of AQP3 and AQP9 both in Healthy donors and Septic patients PBMC and neutrophils.** (A) Histogram summarizing AQP3 and AQP9 levels of expression in healthy donors between genders. (B) Histogram summarizing AQP3 and AQP9 levels of expression in septic patients divided by genders. (C) Histogram comparing the levels of expression of AQP3 and AQP9 between Healthy donors and Septic patients. (D) Representative agarose gel highlighting the expression profiles of septic patients and healthy donors AQP3 and AQP9, β-Actin is used as internal control and reference gene for relative expression analysis. Student's T-test was conducted to compare levels of expression of AQPs between groups; ** $p < 0.01$.

After collecting all the data, in collaboration with statisticians and informaticians, we moved on analyzing the correlations between the patient parameters collected with their transcriptional expression of *AQP3* and *AQP9* detected in PBMC and neutrophils.
Interestingly, *AQP3* mRNA expression correlated ($p < 0.05$) with the blood levels of Procalcitonin (PCT) an inflammatory state marker which has been proven to be able to differentiate only between Sepsis and SIRS of non-infectious origin with a ROC-curve of 0.85 (Wacker, Prkno, et al. 2013).
On the other hand, AQP9 showed a significant correlation with the Septic score NEWS2, major septic score used in combination with the SOFA score to assess the risk of sepsis in patients with suspected infection.

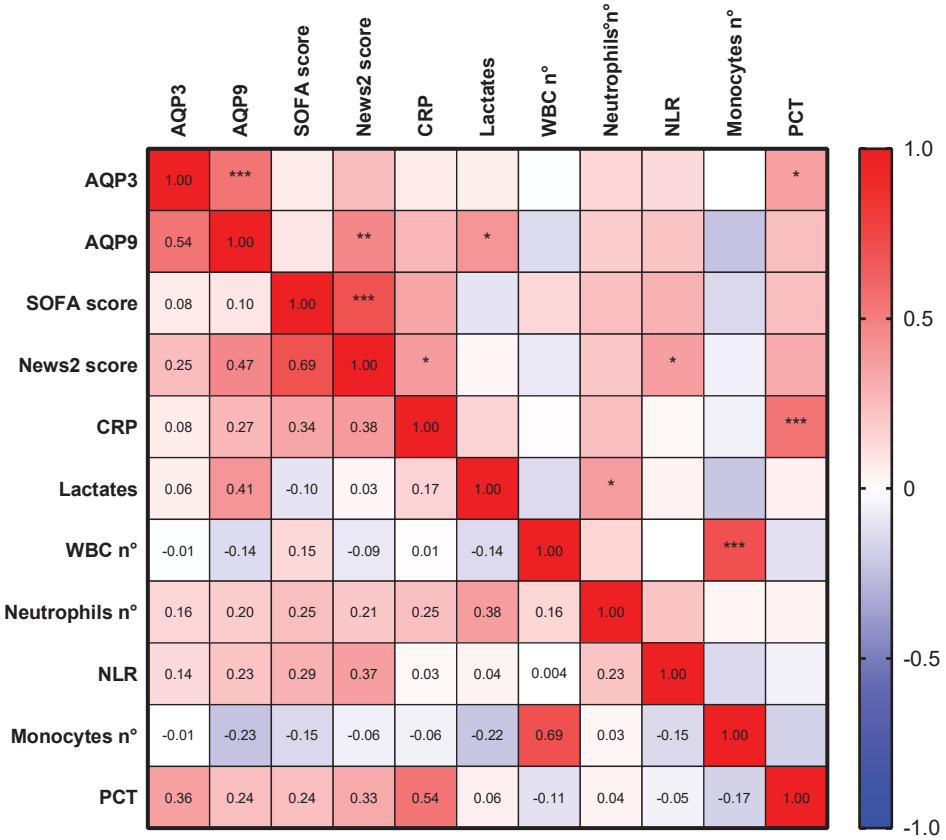
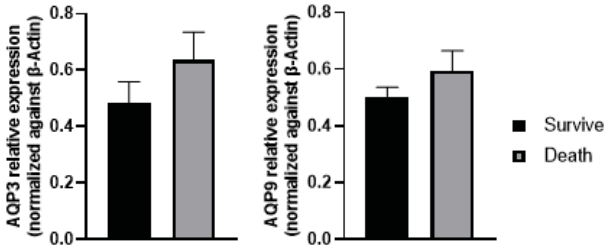


Figure 13 – **Correlation matrix computed between Aquaporins levels of expression, major septic scores and inflammatory markers used in diagnosis of sepsis.** Pearson's correlation factors computed between pairs of variables are shown in the left-half of the matrix while significance levels are shown in the right-half (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Further analyses were conducted to assess whether AQPs expression levels could be of prognostic relevance to predict 28-day mortality in our cohort of patients.
Patients were divided into two groups based on their clinical outcome and interestingly AQP3 showed a higher expression, even if not statistically significant, when compared to the patients that survived, on the other hand, almost the same level of expression was detected across the two groups for the AQP9 expression levels.

A



B

Death	No (N° = 35)		Yes (N° = 12)		
	Average expression	SEM	Average expression	SEM	P-value
AQP3	0.4840	±0.0745	0.6361	±0.0988	0.0557
AQP9	0.5034	±0.0332	0.5949	±0.0709	0.2815

Figure 14 – **AQP3 and AQP9 expression levels stratified based on patient outcome.** (A) Histogram comparing levels of expressions of AQP3 and AQP9 between patient sub-groups. (B) Table summarizing average expression detected in patients. Student's T-test was conducted to compare levels of expression of AQPs between patient sub-groups

With the data collected we trained our first iteration of our ML-based algorithm, Random Forest classification algorithm was chosen due to its robustness against missing values when compared to logistic regressions Algorithms.

For sepsis diagnosis only AQPs expression levels were included, with a computed AUC on the test dataset of 0.76, moreover the algorithm was also trained, by the addition of PCT protein serum levels, on providing 28-day mortality prognosis scoring an AUC on the test dataset of 0.86.

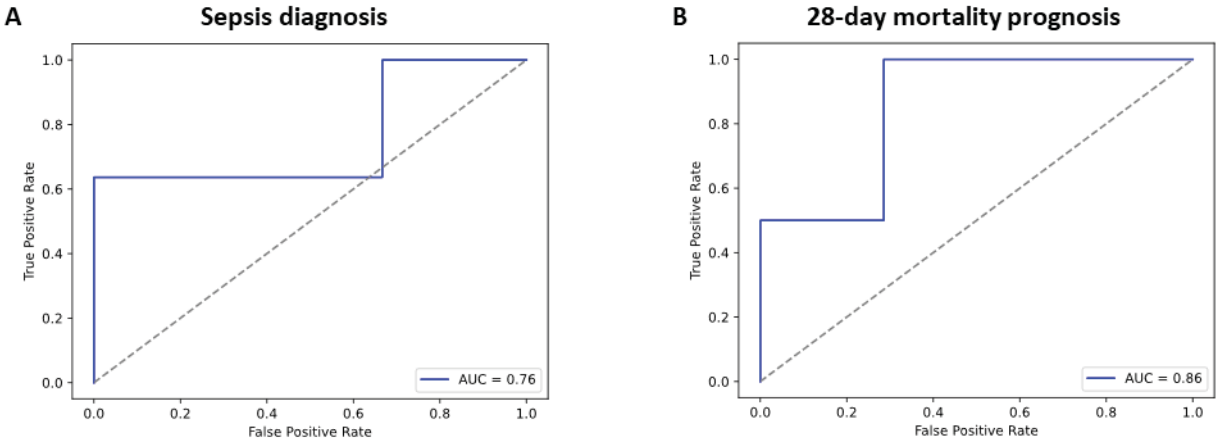


Figure 15 – ROC curve analysis of our random forest classifier for sepsis diagnosis and 28-day mortality prognosis. (A) ROC-curve of sepsis diagnosis using transcriptional expression of AQP3 and AQP9 combined, AUC = 0.76; (B) ROC-curve for 28-day mortality prognosis using a combination of transcriptional expressions of AQP3, AQP9 and blood serum levels of Procalcitonin protein.

II.2.5.3. DFP00173 is not cytotoxic at effective inhibitory concentration

Among the currently implied cell models used to mimic immune cells functions, JURKAT cells represent an AQP3 expressing cell line which could be implied in functional assays to test AQP3 involvement in PBMC cells functions to prove its gating as a possible pharmacological strategy.

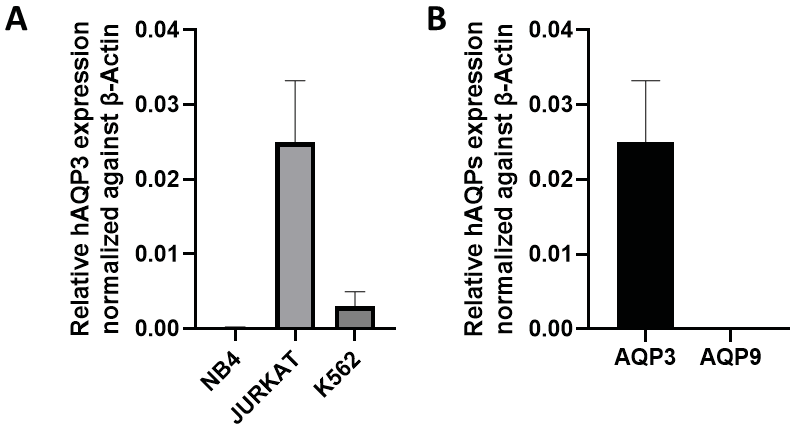


Figure 16 - **Histograms summarizing the expressions levels of Aquaporin-3 in Jurkat, K562 and NB4 cell lines.** (A) RT-qPCR analysis evaluating the expression levels of *AQP3* in three immune cell lines, NB4, JURKAT and K562. (B) RT-qPCR analysis evaluating the expression levels of *AQP3* and *AQP9* in the JURKAT cell line

Based on the data shown above, we have also evaluated the expression and the localization of Aquaporin-3 in JURKAT cells by immunofluorescence. Based on the images shown we could clearly see strong immunostaining localized at the plasma membrane of these cells.

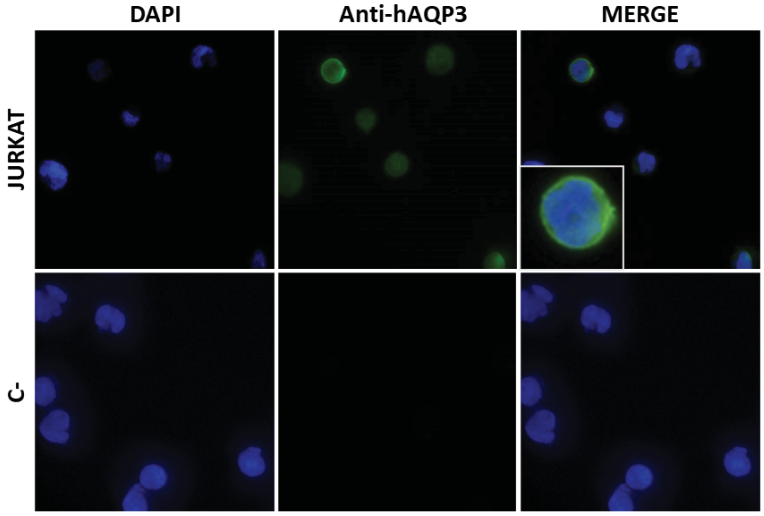


Figure 17 - **Immunofluorescence analysis of expression and localization of hAQP3 in JURKAT cells.** Immunofluorescence images showing a strong signal on the plasma membrane of the JURKAT cells. The antibody used was an Abcam anti-hAQP3 (ab125219) with a dilution of 1:200, C-, negative control consisting of Jurkat cells without incubation with the anti-hAQP3 antibody.

In collaboration with Prof. Graça Soveral (Faculty of Pharmacy, University of Lisbon), we investigated whether the AQP3 inhibitor DFP00173 was involved in any cytotoxic effect on the JURKAT cell line for further studies on PBMC permeability.

Firstly, we excluded any significant cytotoxicity of 0.5% DMSO, the solvent of the inhibitory compound. We found that the compound did not lead to any significant cytotoxicity effect ranging from

1.25 μM – 10 μM, ranges above the computed IC_{50} ~ 0.1 μM (Sonntag et al. 2019). From these results the inhibitor was then used at a concentration of 10 μM (Fig. 18).

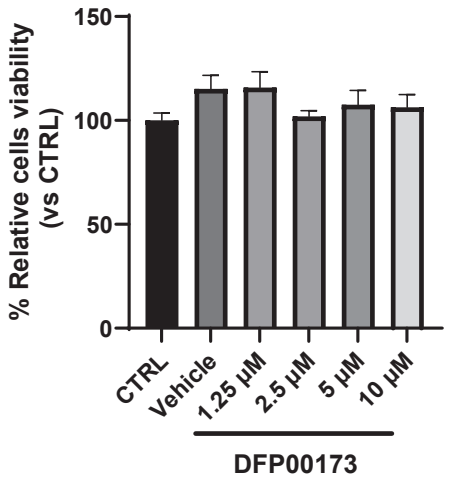


Figure 18 – **DFP00173 cytotoxicity evaluation in JURKAT cells by MTT assay.** Formazan absorbance was measured for each well treated with or without different concentrations of DFP00173. Data points are shown as percentage ratio vs CTRL. CTRL, without inhibitor or vehicle; Vehicle, 0.5% (v/v) DMSO.

II.2.5.4. AQP3 in hydrogen peroxide permeation across plasma membrane

To confirm the involvement of AQP3 in H_2O_2 transport in the JURKAT cell line as a model of human PBMC functional studies were carried out using the AQP3 inhibitor DFP00173.

Previous studies reported that DFP00173 can reduce the hydrogen peroxide permeability in CHO heterologous expressing human AQP3 (Sonntag et al. 2019).

To study if inhibiting AQP3 could affect the H_2O_2 permeation, cells were treated with the specific inhibitor and then exposed to 300 μM H_2O_2 solution. By fitting the resulting data, we measured the slope of the kinetic correlated to the H_2O_2 uptake, observing a significant inhibition of the cell permeability to the hydrogen peroxide by blocking the Aquaporin-3. However, further analysis is needed to assess whether the vehicle alone (DMSO 0.5% v/v) could somehow interfere with the measurements of H_2O_2 permeability (Fig. 19).

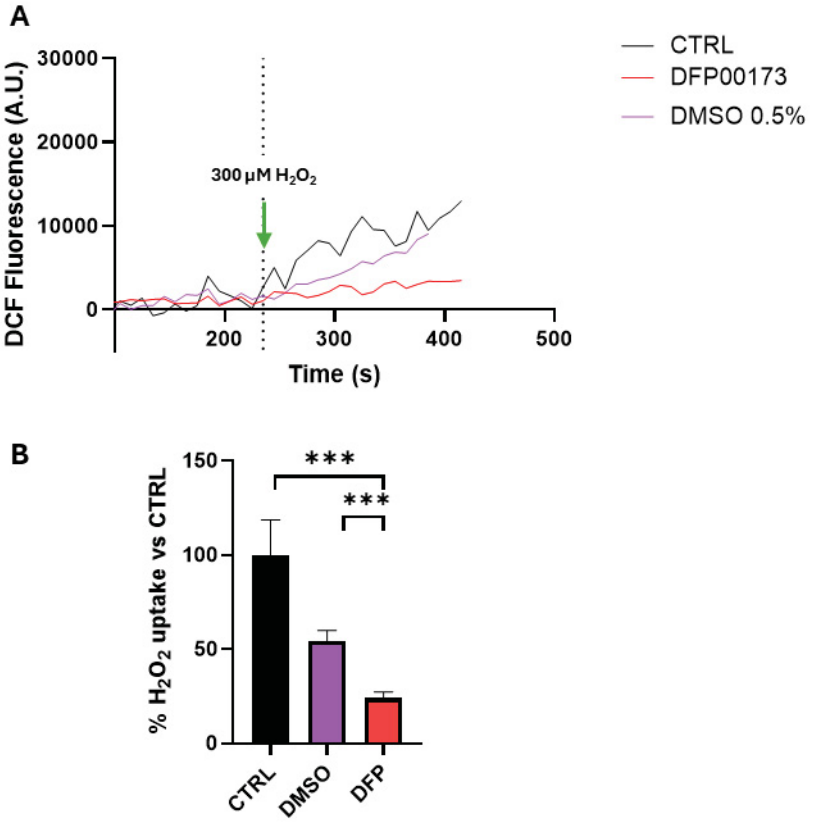


Figure 19 – **Reduction of JURKAT cell H_2O_2 permeability by DFP00173.** (A) Representative time course of H_2O_2 intake after incubating cells with 10 µM DFP00173 inhibitor of AQP3. (B) Hydrogen peroxide permeability of cells in different conditions. (n = 5). Ordinary One-Way Anova ***: P-value < 0.001

II.2.6. DISCUSSION

Sepsis and its most severe complication, septic shock, represent the major challenge for emergency physicians, having an in-hospital mortality rate above 40%.
Antimicrobial therapy is the first crucial step in treating sepsis, in fact, particularly in cases of septic shock, it has been shown that every hour of delay in microbiological sampling is associated with a significant increase in mortality (Ferrer et al. 2014; Kumar et al. 2006).
Microbial culture, the gold standard for sepsis diagnosis, is time consuming, and not all bacteria can be cultured, leading to the delay of diagnosis. The diagnosis of sepsis in critically ill patients is a challenge, as conventional infection markers are often misleading. Therefore, new technologies are being considered, such as omics-based technologies for the identification of immune profiles for the diagnosis and prognosis of sepsis. Unfortunately, these technologies are difficult to use in clinical practice due to their complexity and the amount of work involved (Balc et al. 2003; Cajander et al. 2024).
Since AQPs are reported to exert important role in immune cells, our work concentrated on evaluating the efficacy of AQPs expression levels as easily analyzable predictors able to be implemented in prognostic and diagnostic algorithms needed to ease the screening processes which every suspected patient undergoes.
Firstly, we recruited 47 septic patients which were classified and evaluated to assess possible differences between categorical variables to define sub-groups of patients, nevertheless no sub-grouping was needed based on patient genders or ages. Based on the recorded bedside data and clinical parameters, septic scores (nowadays enrolled in sepsis management) were calculated, needed to stratify septic patients based on their risk of developing sepsis and/or septic shock.
Meanwhile, from the same blood samples collected from patients, we evaluated also *AQP3* and *AQP9* gene expression levels, comparing those levels to the healthy donors.
We carried out semi-quantitative RT-PCR analysis showing a 30% increase of *AQP3* expression levels in septic patients when compared to the healthy donors, while *AQP9* did not show any change in expression.
Notably, by computing Pearson's correlation coefficients between clinical parameters and *AQPs* expression levels in our cohort of septic patients we highlighted a significant correlation between *AQP9* expression levels and News-2 septic score, moreover those expression levels significantly correlated with Lactates levels detected by blood sampling, which have been reported to be highly valuable in predicting 28-day mortality in septic patients (Lee et al. 2021).
Recent studies highlighted a role for Lactates during inflammation, reporting roles triggering neutrophils NET formations, by inhibiting Glycolysis and stimulating oxidative stresses, needed to fight against microbial agents (Caslin et al. 2021).
Being reported in literature that lactate could permeate through AQP9 (Geistlinger, Schmidt, and Beitz 2022), could be reasonable to think that AQP9 might be relevant for neutrophils as an escape-route for lactates and ROS metabolic byproducts to exert their fighting functions against infecting microbial agents.
On the other hand, *AQP3* significantly correlated with the Procalcitonin (PCT) concentrations detected in blood, a novel proposed biomarker implied to screen for severe infections in patients.

To further characterize the prognostic value of AQPs expression levels we compared the expression levels between groups of patients divided based on their clinical outcome during the first 28 day of hospitalization.
Interestingly *AQP3* showed a 25% increase of expression level ($p = 0.0557$) in died patients when compared to the one which survived, nevertheless more studies are needed to expand the patients pool, highlighting the value of AQP3 as prognostic marker for mortality in septic patients.
The AUC-scores of our Random Forest classifier displayed a high performance of *AQP3* and *AQP9* expression levels as predictors of sepsis, while the combined evaluation of AQPs expressions and PCT led to higher performances in providing 28-day mortality prognosis. Altogether these promising results lead us to think that AQP3 and AQP9 might be proven useful as prognostic and diagnostic markers in combination with other sets of biomarkers.
The results achieved in our study highlight the value of AQP expression levels in both neutrophil and PBMC as predictors and possible drug target in sepsis, here the comparison between healthy donor and septic patient allowed us to devise a diagnostic and prognostic algorithm using patient data gathered since the first day of hospitalization.
Recent studies conducted by Thon and coll. also explored the value of AQP3 and AQP9 expression directly from septic whole blood samples of septic patient (Thon et al. 2024). Their analysis relied on the evaluation of both AQP3 and AQP9 gene expression directly from whole blood samples during two different time points for each patient, 1-day and 8-day after sepsis diagnosis.
Their analysis highlighted an up-regulation of AQP3 after 8 days of sepsis diagnosis while AQP9 levels were unaltered in diagnosed septic patients. Moreover, they focused then on the evaluation of AQPs expression in patients 8-day after sepsis diagnosis, showing that patients with higher expression of AQP3 and/or lower expression of AQP9 had better survival chances.
Here, our data appears to be in contrast with what has been reported in the literature, nevertheless major key aspects regarding the cohort composition and the analytical strategy must be taken into consideration since our analytical strategy relied on evaluating AQPs expression as soon as the patient was hospitalized. Considering the findings from Thon and coll., further evaluation will be carried out, expanding our cohort of patients, evaluating the levels of expression of both AQPs during different hospitalization timepoints.
Of note, the hydrogen peroxide entering the PBMC may be important for the activation of the inflammatory pathways (Surabhi et al. 2022), to this point, the commercially available inhibitor of AQP3, DFP00173, is a useful tool to investigate the permeability properties of AQP3 in PBMC which might be established as a drug target in various infectious and non-infectious diseases, but further evaluation are still needed as will be discussed in the third chapter.
Being able to elucidate the different functions of AQP3 and AQP9 in PBMC and neutrophils we might be able to propose novel pharmacological strategies to treat sepsis and septic shock by selectively blocking both AQPs.

II.2.7. CONCLUSION

The findings presented in this chapter underscore the potential of AQP3 and AQP9 as diagnostic and prognostic biomarkers in sepsis. We found a significant increase (30%) in AQP3 gene expression in the PBMCs of septic patients compared to those of healthy donors. The expression levels of AQP3 and AQP9 correlated with critical clinical parameters such as the NEWS-2 score, serum lactate levels, and serum procalcitonin concentrations. These results suggest that the joint evaluation of AQP3 and AQP9 may serve as a valuable indicator for risk stratification and outcome prediction in septic patients.

Furthermore, integrating the molecular data with clinical parameters through an ML approach based on a random forest classifier further improved the predictive value of both AQPs. The results achieved pave the way for the development of innovative diagnostic tools that could support clinical decision-making in ICU context. Although preliminary, these results represent a step toward personalized medicine and highlight the need for further studies to validate and optimize these predictive ML-based models.

Chapter 3

The human promyelocytic NB4 cell line as an in vitro model to investigate the roles and regulation of AQP9 in neutrophils

II.3.1. ABSTRACT

Inflammatory disorders pose a major challenge in medical research, requiring robust models to investigate molecular pathways and evaluate potential therapeutic strategies.
For decades, murine models have been the gold-standard platform to test for therapeutic targets and gaining molecular insights into inflammatory disease pathways, despite the extensive use of these models it has been proven that these platforms poorly mimic human inflammatory disorders, posing a challenge in understanding the molecular pathways that undergo during inflammation. Moreover, multiple clinical trials drugs failed in human testing while were suggested as promising therapeutical strategies when tested in murine models.
Here, human immortalized myeloid cell lines such as THP-1, U937, HL-60, and NB4 might serve as valuable tools to study inflammatory responses to noxious stimuli, including cytokine production, leukocyte migration, and pathogen interactions, addressing the limitations associated to the usage in culture of blood isolated immune cells. Hence, our goal in this chapter is to test and characterize AQPs expression in NB4 cells, an Acute Promyelocytic leukemia (APL) cell line, capable of being stimulated to differentiate into mature neutrophils, to prove them as a powerful tool to test AQPs involvement in neutrophils functions in both physiological and pathophysiological conditions.
Flow Cytometry analysis was carried out to test the expression of typical neutrophilic cell-surface markers, such as the CD-15, upon stimulation of differentiation.
The expression levels of *AQP3* and *AQP9* mRNA upon treatment with differentiating compounds were assessed through RT-qPCR comparing expression levels between differentiated cells and their circulating counterparts, showing that the combined treatment with All-Trans retinoic acid (ATRA) and Granulocyte-colony stimulating factor (G-CSF) led to a significant increase in *AQP9* expression with a significant reduction of *AQP3* mRNA after 5 days of differentiation when compared to the basal cells. Immunofluorescence and Immunoblotting analysis were also conducted to visualize the expression and localization of AQPs upon differentiation.
Despite the upregulation seen for AQP9, its levels of expression in differentiated cells remain 100-fold lower than their circulating counterparts.
Other than immune cells functions, AQPs molecular transport might also be relevant during myeloid differentiation processes where AQP9 might play a role during metabolic shifts in neutrophil-committed myeloid cells by maintaining cellular homeostasis, facilitating the export of metabolic by-products such as lactates and ROS.
The experiments of phosphorylation profiling of cell signaling pathways run in presence or absence of a potent and selective inhibitor of AQP9, RG100204, suggest a role for this AQP in cell migration, cell adhesion and inflammatory response as indicated by the changes in the extent of phosphorylation of PDGFRβ, FGR, and members of the STAT family upon cell exposure to LPS, TNFα, IL-6 and fMLP. This is consistent with previous observations by our laboratory and other authors.
Taken together, these results provide a useful starting point to define the precise molecular mechanisms that involve AQP9 in the pleiotropic actions that this AQP appears to perform in mature neutrophil granulocytes. Appropriately engineered to express AQP9 natively, NB4 cells could represent a valuable human-derived platform to analyze the modulation and biophysical properties of AQP9 and test novel neutrophil-targeted therapies involving AQP9.

II.3.2. INTRODUCTION

II.3.2.1. In vitro and in vivo models for studying inflammatory disorders

The path to describe novel therapeutic approaches for inflammatory disorders is related to the evaluation of the efficacy of therapeutic molecules through *in-vitro* assay.

Most of the research fields make use of different kinds of immortalized cell lines to study a wide variety of diseases and their associated pathways.

Immunological research is a unique field of study due to the intricate complexity and the dynamism of human immune systems, in fact, unlike other biological systems, immune system is composed by a vast number of different cell types, signaling molecules, pathways and different kind of tissues and organs.

Different *in vivo* and *in vitro* models have been proposed to screen and evaluate the efficacies of novel therapeutical approaches for inflammatory diseases, most of the studies rely on the systemic inflammatory effects, focusing directly on animal models as in vivo sources of information (Bernardette Martinez-Rizo et al. 2024).

It is well known that monocytes and macrophages play key roles during inflammatory responses, which are related to the several cell lines resembling them derived from both humans (such as the THP-1 and U937 cell lines) and mice (like RAW264.7 and J774).

THP-1 and U937 cell lines have been extensively used by researchers to gain insights into the inflammatory pathways since they are able to mimic both monocyte or macrophages upon stimulation, by mimicking their circulating counterparts functions, structures, mechanisms and signaling pathways (Chanput, Mes, and Wichers 2014; Zhang et al. 2018).

Various stimuli can be used to trigger inflammatory responses on these *in vitro* models, such as INF-γ, IL-1β, TNF-α and LPS, with the LPS being the most common inflammatory trigger recognized by the TLR-4 receptor, leading to the activation of the NF-κB and MAPK pathways, inducing the production of pro-inflammatory cytokines (He et al. 2012; Yang et al. 2016).

Given that inflammatory processes involve also leukocyte migrations, the U937 cell line is commonly used to evaluate and modulate monocytes and macrophage's ability to attach to extracellular matrices, extravasation and migration capabilities (Blasi et al. 2011).

Other component of the immune system which is gaining a lot of attention are represented by Granulocyte neutrophils, these cells play a major role in diseases such as infection, autoimmunity and cancer but, due to their short lifespan and their inability to proliferate in culture (Coffelt, Wellenstein, and de Visser 2016), they still pose a challenge for studying their functions and pathways (Cockx et al. 2018; De Bondt et al. 2020; Rapoport et al. 2020).

Nowadays, Neutrophil research mostly relies on cells isolated from healthy donor peripheral blood, which introduces donor-dependent variability in the experiments, to counteract these problems different cell models have been proposed.

The most commonly used cell line used to mimic neutrophils functions is the HL-60 cell line, deriving from a female patient with Acute Promyelocytic Leukemia (APL) (Birnie 1988; Newburger et al. 1979), which can undergo different genetic editing techniques (Bao et al. 2019; Narimani, Sharifi, and Jalili 2019) and most importantly, can be differentiated in Neutrophil-like cells by treatment with All-Trans Retinoic Acid (ATRA).

Despite their ability to mimic neutrophils after ATRA differentiation these cells have been proven to be able to recreate the NETosis (Wang et al. 2009) and the ROS production (Kitagawa et al. 1984) of Peripheral neutrophils as well as phagocyte pathogen, without being able to intracellularly kill it due

to the lack of specific granules (Chen, Wu, et al. 2014; Matzner et al. 1987; Newburger et al. 1979; Roberts and Ford 1982).
NB4, a relatively less characterized cell line derived from Acute Promyelocytic Leukemia, has traditionally been employed to study cell differentiation mechanisms in leukemia. Recently is gaining a lot of attention due to its ability to produce specific granules upon ATRA differentiation, leading to a better platform for studying neutrophils functions in contrast to HL-60 cells (Lanotte et al. 1991; N'Diaye et al. 1997).
Despite the advances in suitable *in vitro* models, which are clearly feasible for studying intracellular pathways, the gold-standard for studying complexes inflammatory pathways and novel drugs is still represented by mice models.
Based on the field of interest, different kind of experimental models can be used, starting from the carrageenan induced paw edema which causes an acute nonimmune inflammation (Archer, Muthukumar, and Halami 2015), it is induced by a sub plantar injection in the mice paw of 1% λ-carrageenan, leading to edema formation and neutrophil infiltration, increasing the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α as well as Reactive Oxygen Species (ROS) production.
The main advantage given by this kind of approach lies in its biphasic response to the injection, giving the opportunity to evaluate the biological target responsible for the anti-inflammatory effect.
As a subset of inflammatory disorders, Sepsis has been the most widely studied for which different kinds of *in vivo* models have been proposed and tested.
The most used model to study inflammatory pathways is the intra-peritoneal LPS injection in mice causing endotoxemia which resembles human sepsis (Deitch 2005).
Since this kind of model relies on LPS injections, a component of Gram-negative bacteria wall, its effectiveness is well established in understanding the TLR-4 (receptor for the LPS) signaling pathway on an organism scale (Chen, Stanojcic, and Jeschke 2014; Rittirsch, Hoesel, and Ward 2007) this kind of model neglects the host-pathogen interaction, rising questions in the scientific community whether these injected mice might only be used as a platform to test novel therapeutic strategies for sepsis (Remick et al. 1995; Remick and Ward 2005; Rittirsch, Hoesel, and Ward 2007).
To overcome these limitations, nowadays a novel sepsis model is widely used known as CLP (Cecal Ligation and Puncture) mice, which involves the ligation of the mice cecum and its perforation by needle puncture (Baker et al. 1983; Wichterman, Baue, and Chaudry 1980), leading to a polymicrobial sepsis from fecal spillage after the puncture (Dejager et al. 2011).
This CLP model represents the most coherent model with the human counterpart, since it results in a concomitant activation of pro-inflammatory and anti-inflammatory pathways (Dejager et al. 2011), moreover, the ischemia derived from the ligated tissues contributes to the immune system impairment observed in sepsis cases (Ayala et al. 2000).
Despite the effectiveness of these mice models on gaining insights in molecular pathways during inflammatory disorders on a larger scale, there are limitations that need to be taken in account, above all the timing and the response magnitude of the immune system to the septic insults, which are different between mice and humans (Rittirsch, Hoesel, and Ward 2007; Lewis et al. 2016; Remick et al. 2002), which has been defined, by the scientific community, the cause that led the anti-TNF antibody therapy to fail during clinical human trials, after being promising when tested in mice models (Remick et al. 1995).

II.3.2.2. The Acute Promyelocytic Leukemia (APL) NB4 cell line

NB4 cell line is promyelocytic cell line established from a 20 year old woman with acute promyelocytic leukemia (APL) firstly established in 1989 and described in 1991 by Dr. Lanotte and colleagues (Lanotte et al. 1991).

Unlike other leukemia-derived cell lines, NB4 cells carry a specific cytogenetic abnormality with a balanced translocation (t(15;17)), putative cause of the M3 acute myelogenous leukemia (Lanotte et al. 1991).

The translocation creates a hybrid gene between the C-terminus region of the Retinoic acid receptor-α (RARα) and the amino-terminus of the promyelocytic leukemia locus (PML), creating a PML-RARα fusion gene, which mRNA has been detected in patients with APL (Castaigne et al. 1992).

According to the hypothesis, the PML-RARα gene product represents a dominant-negative oncogene which is capable of disrupting gene pathways involved in the normal development of granulocyte maturation (de The et al. 1991), by forming a transcription repressive complex with the PU.1 transcription factor (Wang et al. 2010), blocking the physiological hematopoiesis.

Retinoic-acid has been proven to play a role in the granulocytic maturation pathway, indeed the administration of all-trans retinoic acid (ATRA) has been used for years to achieve the complete remission in patients with APL (Huang et al. 1988) by degrading the aberrant PML-RARα fusion protein (Weis et al. 1994; Yoshida et al. 1996) and subsequently inducing the differentiation of the cancerous cells (Warrell et al. 1991).

The differentiation of NB4 cells by ATRA compound induces the cells to express also dendritic cell surface markers such as CD80, CD86 and adhesion molecules like CD40 (Zhou and Tedder 1995) via the NF-κB activation pathway (Yoshimura et al. 2001).

For decades, APL derived cell lines have been extensively used by researchers to gain insights into the pathophysiology of leukemic malignancies as well as therapeutical strategies to selectively target cancerous cells.

II.3.3. AIM OF THE STUDY

The main objectives of the work run in this chapter were: (i) to assess the expression of aquaporins in a neutrophil-like, inducible cell line derived from human acute promyelocytic leukemia (APL), and (ii) to characterize the response of NB4 cells to various differentiation stimuli, thereby validating the cell line as a reliable model for human peripheral blood neutrophils. With the help of the specific inhibitor RG100204, the NB4 cells stimulated to differentiate in mature neutrophils were then used to investigate the signaling pathways triggered by the molecules moving through the AQP9 channel.

II.3.4. MATERIALS AND METHODS

II.3.4.1. NB4 cell culture

Acute promyelocytic leukemia NB4 cells (CVCL_0005) were cultured in RPMI-1640 w/o L-Glutammine (Biowest, Nuaillè, FRANCE) + 10% FBS (Gibco, Grand Island, New York, USA) + 10 U/mL Penicillin and 0.1 mg/mL Streptomycin (Euroclone, Milan, ITALY).
Before passaging, cells mantained until reaching a concentration of $2x10^6$ cells/mL.
Upon reaching optimal concentration, cells were splitted in T75 flasks and media was changed with fresh complete RPMI-1640 + 1 µM All-Trans Retinoic Acid (Merck Millipore, Burlington, MA, USA) + 0.01% DMSO (Carlo Erba, Milan, ITALY) to stimulate differentiation in Neutrophil-like cells, as a vehicle control, another batch of cells were resuspended in fresh complete medium + 0.01% DMSO, media was changed after 2/3 days of culture.
For each timepoint (T1, T3, T5) of ATRA treatment, an aliquot of cells was withdrawn from the flask and used for later analysis.

II.3.4.2. Flow-Cytometry analysis of differentiated NB4 cells

To examine the expression of cell surface markers, we used the Flow-Cytometry, thanks to Dr. Franceso Albano from the Dimepre-J (University of Bari Aldo Moro).
Basal and ATRA/DMSO-treated cells were sedimented by centrifugation for 300xG for 5 minutes, washed with FACS-Buffer (0.5% BSA +2 mM EDTA in PBS) and then resuspended in PBS with anti-CD15-FITC conjugated antibody (BD™ CD15 FITC, #332778, BD™ Biosciences) and anti-CD117-PE conjugated antibody (BD™ CD117 PE, # 332785, BD™ Biosciences).
CD15 monoclonal antibody, conjugated with the FITC-a fluorochrome (495/519 nm), was implied to assess the presence of the Neutrophil specific marker on the cell membrane of differentiated NB4 cells while CD117 monoclonal antibody, conjugated with PE fluorochrome (480/578 nm) was used to assess the presence of undifferentiation markers on the cell membrane.
The flow cytometry results were analyzed using the FlowJo™ v10.8 software (BD Life-Sciences).

II.3.4.3. Total RNA extraction from basal and differentiated NB4 cells

Total RNA extraction was performed using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol.
After each timepoint, cells were counted and $1x10^6$ cells were withdrawn for RNA extraction.
Cells suspension was centrifuged, and supernatant was discarded, cell pellet was then lysed by resuspending in 750 µL of TRIZOL (Thermo Fisher Scientific, Waltham, MA, USA), later, 200 µL of Chloroform were added and mixed by shaking for 15 seconds.
Subsequently, the mix was centrifuged at 12000xG for 15 minutes at 4°C using the Phasemaker™ Tubes (Thermo Fisher Scientific, Waltham, MA, USA), enhancing the aqueous phase collection.
To the aqueous phase were added 1.5 volumes of 100% ethanol and were filtered through the spin column by centrifugation at 8000xG for 15 seconds to allow total RNA to bind to the column.
After binding the RNA to the column, it was washed and then eluted with 30 µL of RNAse free water.

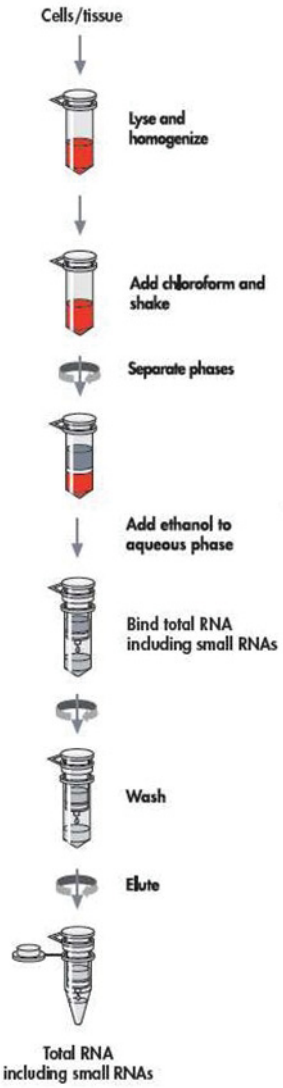


Figure 20 – **Flow chart of total RNA extraction phases from stimulated and non-stimulated NB4 cells.** Upon cell lysis, RNA suspension was filtered through the spin column membrane to allow its separation, then it was eluted by centrifugation of the membrane with Nuclease-free Water

II.3.4.4. Semi-Quantitative analysis of AQP3 and AQP9 gene expression in NB4 cells

To determine gene expressions levels of AQP3 and AQP9 in basal NB4 cells, ATRA-treated cells and neutrophils, we employed a semi-quantitative RT-qPCR analysis.

Firstly, 400 ng of previously extracted total RNA was retrotranscribed in cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA).

Reactions were prepared as described below:

RT-Master Mix	Volume (µL)
10x RT-Buffer	2.0
25x dNTP Mix (100 mM)	0.8
10x RT Random Primers	2.0
Multiscribe Reverse Transcriptase	1.0
RNase Inhibitor	1.0
RNA + H_2O	Up to 20 µL of total Reaction
Total Volume of reaction	20 µL

Samples were then placed in a in a MiniAmp Thermal Cycler (Applied Biosystems™, Waltham, MA, USA) with the following program:

Step	Temperature	Time
RNA denaturation	25°C	10 min
cDNA polymerization	37°C	120 min
Transcriptase degradation	85°C	5 min

After cDNA synthesis, each sample was quantified via Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and qPCR reactions for AQP3 (5' - GGAATAGTTTTT-GGGCTGTA -3'; 5' - GGCTGTGCCTATGAACTGGT – 3'), AQP9 (5' - ATGTGGGAGCCCAG-TTCTTG - 3'; 5' - TACGGAGCTGGGTATGTTGC - 3') and β-Actin (5' - CTGTGGCATCCAC-GAAAC – 3'; 5' - CAGACAGCACTGTGTTGG -3') were carried out using the PowerUp™ SYBR™ Green Master Mix for qPCR (Applied Biosystems™, Waltham, MA, USA) as follows:

qPCR-Master mix	Volume (µL)
PowerUp™ SYBR™ Green Master Mix (2X)	5.0
Forward and reverse primers 8 µM (10X)	1.0
Nuclease-Free Water	3.0
cDNA template (50 ng)	1.0
Total volume of reaction	10.0

Samples were plated in duplicates and loaded in a StepOnePlus™ Real-Time PCR System (Applied Biosystems™, Waltham, MA, USA) with the following program:

Step	Temperature	Time	Cycles
UDG activation	50°C	2 min	1
Activation (Dual-Lock™ DNA polymerase)	95°C	2 min	1
Denature	95°C	15 sec	40
Annealing	56°C	15 sec	
Extension	72°C	30 sec	

To prove the specificity of amplification, melting curves were performed after the last cycle using the program below:

Step	Ramp rate	Temperature	Time
1	1.6°C/sec	95°C	15 sec
2	1.6°C/sec	60°C	1 min
3	0.2°C/sec	95°C	15 sec

Relative expression for *AQP3* and *AQP9* was calculated using β-Actin as reference housekeeping gene and Basal expression levels as calibrators according to the $2^{-\Delta\Delta Ct}$ method.

II.3.4.5. Immunofluorescence analysis of AQP9 expression in NB4 cells

Immunofluorescence was performed to observe the localization of AQP9 in basal and differentiated NB4 cells.

Cells were seeded on glass slides and incubated at 37°C for 7 minutes, then fixed with 4% paraformaldehyde for 20 minutes. Subsequently, cells were washed with PBS and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 20 minutes.

Cells were blocked in 3% BSA in PBS for 60 minutes at room temperature, then cells were incubated for 60 minutes with rabbit polyclonal anti-AQP9 primary antibody (1:100, #SAB4301752 Merck) diluted in PBS supplemented with 3% BSA. As a negative control, cells were incubated without the primary antibody.

Later, cells were washed with the same block solution and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000, #A-21206, Thermo Fisher Scientific) in PBS+3% BSA for 60 minutes at room temperature in the dark, subsequently slides were mounted and nuclei were stained with Fluoroshield™ with DAPI (Merck).

Slides were observed with the Leica DMRXA fluorescent microscope and images were captured via Leica DFC450 C camera and processed using Leica Application Suite Advanced Fluorescence Software (LAS AF 3.1.0).

II.3.4.6. Cell lysis and Western Blotting

Proteins from basal and differentiated NB4 cells were extracted by radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with Protease inhibitors (#TB264086, Thermo Fisher Scientific) and then quantified via Pierce™ BCA protein assay kit (#23227, Thermo Fisher Scientific).

10 µg of proteins were denaturated by boiling for 5 minutes at 90°C, then protein samples were separated on a 4-12% polyacrylamide gel (Bolt™ Bis-Tris Plus mini protein gels, #NW04122BOX, Thermo Fisher Scientific) and electro-transferred to a polyvidene difluoride (PVDF) membrane (#IPVH00010, Merck) using a Mini Blot Module (#B1000, Thermo Fisher Scientific).

Membranes were blocked with 5% non-fat dry milk dissolved in Blotto (20 mM TRIZMA base, 150 mM NaCl, 1% Triton X-100) and subsequently incubated overnight at 4°C with our primary antibody rabbit anti-rAQP9 (1:1250).

Proteins were detected via incubation with goat anti-rabbit IgG-peroxidase (1:5000, #A0545, Merck) and subsequent reaction with ECL West Femto Plus Substrate (#ECL-2002, SOCIETÀ ITALIANA CHIMICI DIVISIONE SCIENTIFICA) according to manufacturer instructions.

Membranes were then visualized using a Bio-Rad ChemiDoc.

II.3.4.7. Phosphorylation profiling of stimulated NB4 cells

Phosphorylation profile of proteins was assessed by using the Human Phospho-Kinase Array kit (Biotechne, R&D Systems, Minneapolis, Minnesota, USA).

After differentiation of NB4 cells with the combined treatment of 1 µM ATRA and 10 ng/mL G-CSF for 5 days, cells were then incubated with the AQP9 specific inhibitor RG100204 or with 0.5% DMSO (Vehicle) and subsequently challenged with LPS (100 ng/mL), TNFα (10 ng/mL), IL-6 (10 ng/mL) and fMLP (100 nM). 10^7 cells were lysed by resuspension and rocking for 30 minutes at 4°C in the supplied lysis buffer supplemented with 10 µg/mL Aprotinin, 10 µg/mL Leupeptin hemisulfate and 10 µg/mL Pepstatin A. The suspension was then centrifuged at 14000xG for 5 minutes and the supernatant was collected, the protein suspensions was then quantified through Pierce™ BCA Protein Assay Kits and same amount of protein per condition were diluted to a final volume of 2 mL with Array Buffer 1.

Membranes were incubated with 1 mL blocking buffer for 1 hour according to the manufacturer instruction, then, blocking buffer was then discarded and 1 mL of pre-diluted suspension was added to each corresponding membrane and were let incubate overnight at 4°C on rocking platform shaker.

Membranes were then washed three times for 10 minutes in 1X Wash Buffer, subsequently, each part A of the membrane was incubated with 1 mL of Array Buffer 2/3 supplemented with the Detection Antibody Cocktail A, while part B of the membrane was incubated with 1 mL of Array Buffer 2/3 supplemented with Detection Antibody Cocktail B.

Membranes were then incubated for 2 hours at room temperature on a rocking platform.

After 2 hours, each membrane was washed in 20 mL of 1X Wash Buffer for 10 minutes on a rocking platform shaker.

Membranes were then incubated with Array Buffer 2/3 supplemented with Streptavidin-HRP for 30 minutes at room temperature on a shaker, subsequently membranes were washed three times for 10 minutes each in 1X Wash buffer.

Washed membranes were then positioned in plastic sheet protectors and incubated with the Chemi Reagent Mix for 1 minute then membranes were visualized using a Bio-Rad ChemiDoc.

II.3.4.8. Statistical analysis.

The statistical analysis was conducted through GraphPad Prism software for Windows (version 9.0.0). For the fold change expression of *AQP3* and *AQP9* in differentiating NB4 cells across 5 days of treatment, Two-way analysis of variance, followed by a Dunnet multiple comparison test, was conducted. Fold changes of expression of *AQP3* and *AQP9* in differentiated NB4 cells by different stimuli were compared through one-way analysis of variance, followed by a Dunnet multiple comparison test. Student's T-test were carried out to compare level of expression of *AQP3* and *AQP9* between differentiated NB4 cells and isolated neutrophils. Comparison of phosphorylation levels of proteins were conducted by a multiple Student's T-test. In all cases, a p-value of less than 0.05 was considered significant.

II.3.5. RESULTS

II.3.5.1. Differentiated NB4 cells mimic neutrophil phenotype expressing high levels of AQP9

To evaluate the efficacy of differentiation in Neutrophil- like cells of NB4 cells treated with All-Trans Retinoic Acid (ATRA) we implied Flow cytometry analysis of NB4 cells treated with 1 µM ATRA + 0.01% DMSO or 0.01% DMSO as vehicle control at different timepoints: 1-day after ATRA treatment (T1), 3-days after ATRA treatment (T3) and 5-days after ATRA treatment (T5).

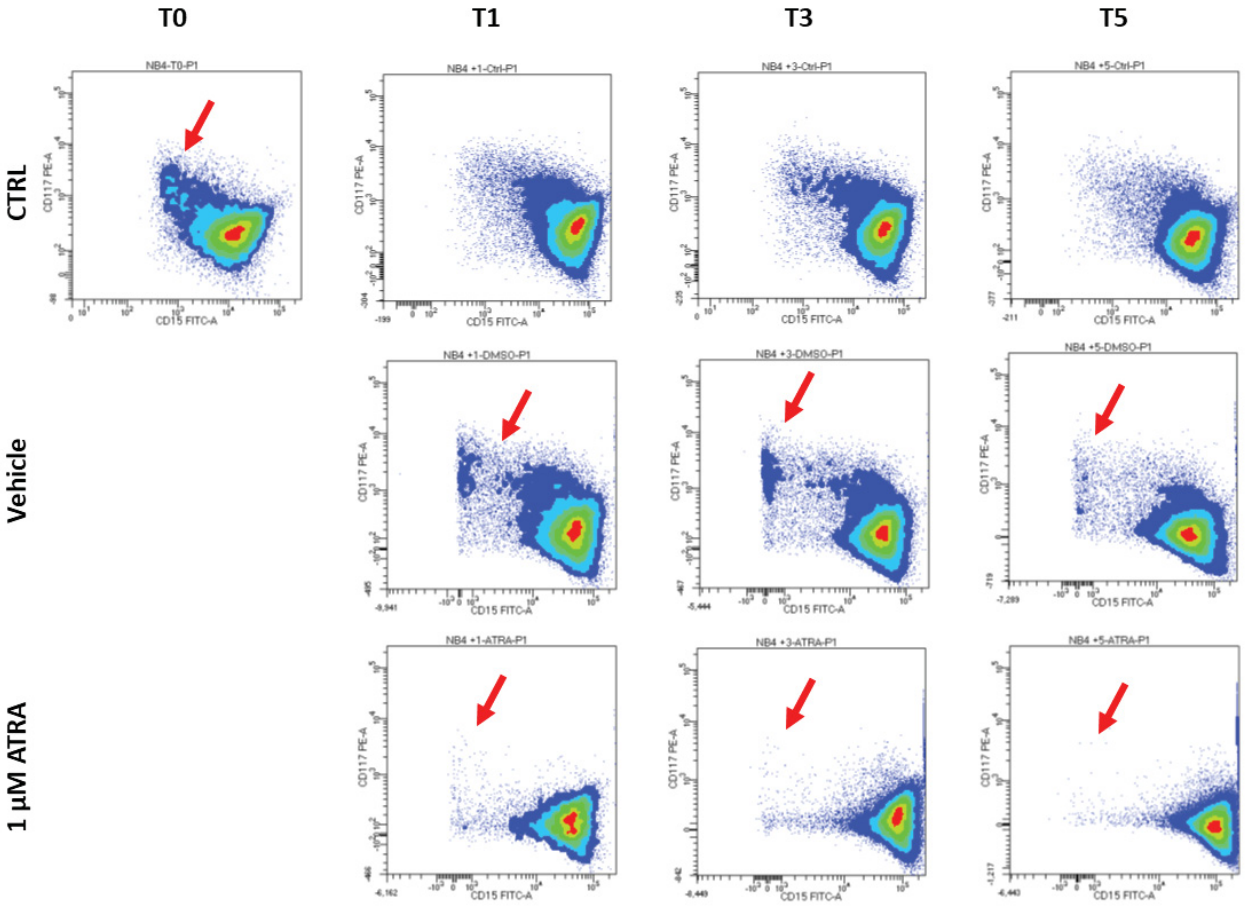


Figure 21 - **Flow cytometry assay for differentiation of NB4 cells after ATRA treatment.** Red arrow represents the cluster of $CD117^{+}$ undifferentiated cells, which clearly shifts towards $CD15^{+}$ cells after exposure to All-Trans Retinoic Acid for 5 days.

Two specific markers were used to follow the differentiation of NB4 cells into neutrophil-like cells, CD15 was used to assess the differentiation in mature neutrophils while CD117 was used as a marker of undifferentiated cells being a specific marker for hematopoietic stem cells (Fig. 21).

As proven by the Flow Cytometry analysis, we were able to differentiate NB4 cells into neutrophil-like cells after 5 days of treatment with ATRA leading us to the evaluation of Aquaporin-3 and Aquaporin-9 gene expression in basal NB4 cells and differentiated NB4 (dNB4).

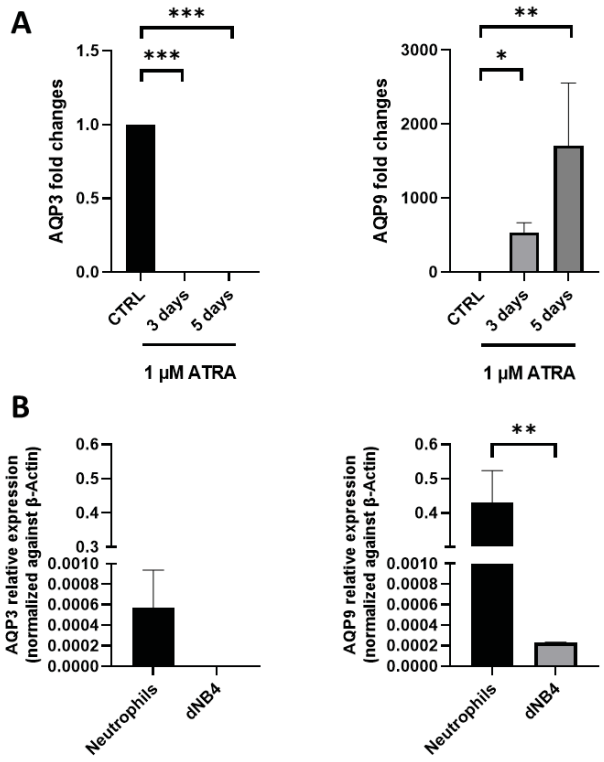


Figure 22 – **RT-qPCR analysis of AQP3 and AQP9 gene expression in ATRA-treated NB4 and relative expression of AQP3 and AQP9 between human neutrophils and differentiated-NB4 cells**. (A) fold changes of AQP3 and AQP9 on NB4 cells with differentiation inducers All-Trans retinoic acid. Two-way ANOVA statistical tests was performed (B) Relative expression of AQP3 and AQP9 comparing expression levels between neutrophils and ATRA differentiated NB4 cells, T-student's test was performed (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

To test AQPs gene expression in NB4 cells and peripheral blood neutrophils we carried out RT-qPCR analysis comparing the expression levels between peripheral blood neutrophils, basal NB4 cells and ATRA-differentiated cells.

Interestingly, AQP3 showed a significant down-regulation in differentiated NB4 cells after 5 days of treatment with ATRA, leading to a total absence of expression of AQP3 (Fig. 22A).

On the other hand, AQP9 showed an opposite trend, with an up-regulation as soon as 3 days after ATRA treatment, with a peak expression after 5 days of treatment (Fig. 22A), nonetheless, when compared with the isolated neutrophils, its level of expression was still 1844-fold less than the levels detected in isolated neutrophils (Fig. 22B).

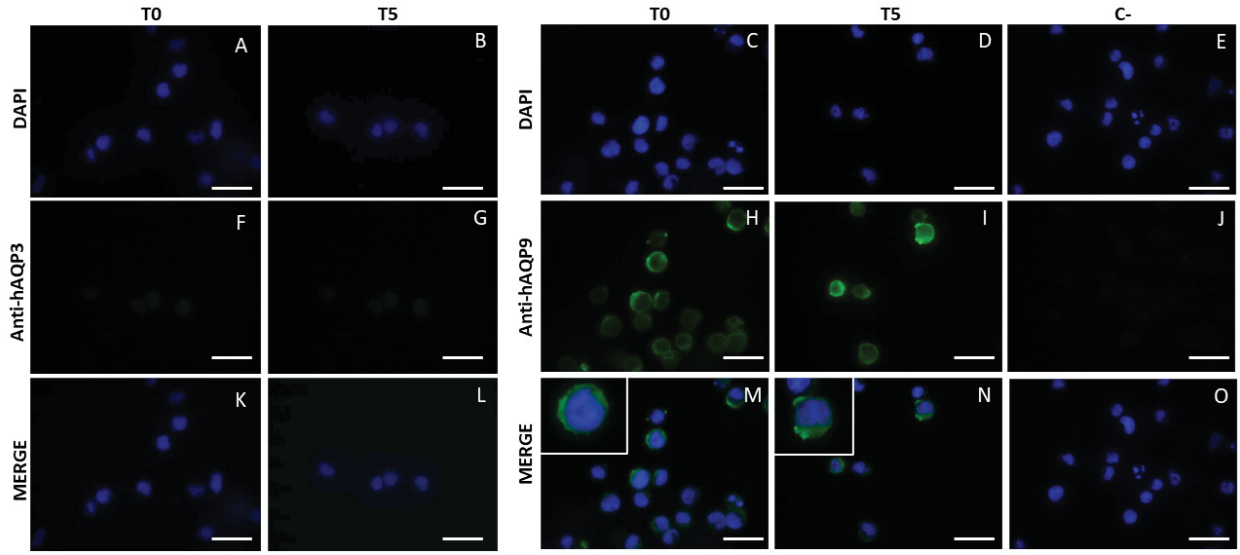


Figure 23 – Immunofluorescence analysis of AQP9 on differentiated NB4 cells. Differentiated cells were submitted to immunofluorescence analysis, nuclei were stained with DAPI (A, B, C, D, E) in blue, AQP3 was labelled with Abcam anti-hAQP3 (ab125219) at a 1:200 dilution (F, G), AQP9 was stained with SIGMA anti-AQP9 (#SAB4301752, Merck) in green (H, I), respectively the channels were merged (K, L, M, N, O), C- : Negative control omitting primary antibody; scale bar: 20 µm

ATRA-differentiated cells were submitted to immunofluorescence analysis to confirm the expression and localization of both AQP3 and AQP9. The analysis confirmed the expression of AQP9 after ATRA treatment, with stronger spots upon ATRA treatment for 5 days (Fig 23), AQP3, on the other hand, remained undetectable even after ATRA treatment.

Being known in the literature that myeloid cells differentiation could be enhanced by synergistic treatments of All-Trans Retinoic acid and Granulocyte Colony stimulating factor (G-CSF) (Maun et al. 2004), we tested the AQP3 and AQP9 expression after the combined effect of ATRA+G-CSF for 5 days.

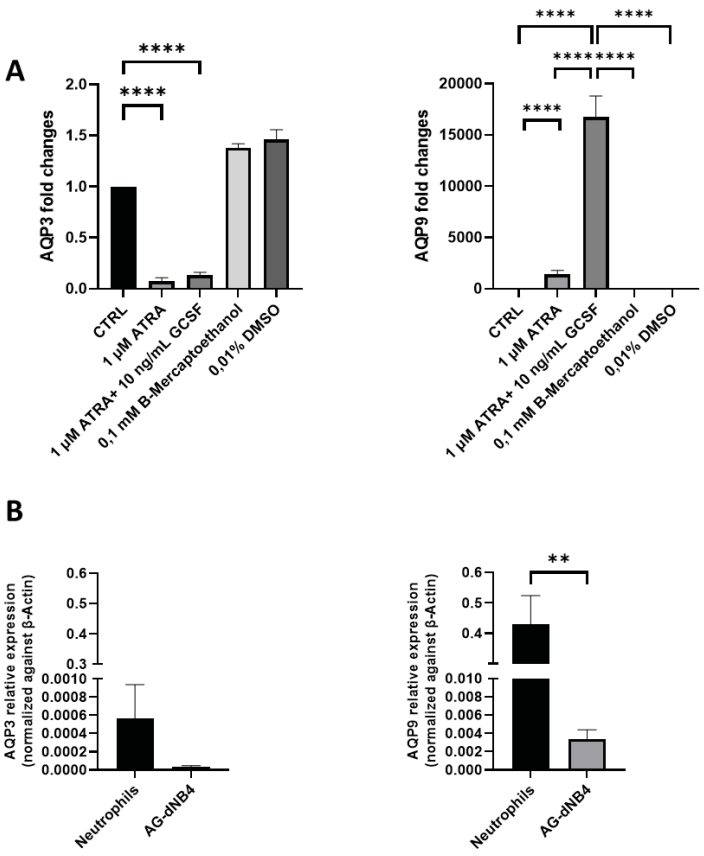


Figure 24 – RT-qPCR analysis of AQP3 and AQP9. (A) fold changes of AQP3 and AQP9 on NB4 cells with differentiation inducers All-Trans retinoic acid alone and ATRA + Granulocyte colony stimulating factor. 0.1 mM β-Mercaptoethanol was kept in the media during differentiation to compensate for oxidative stress during differentiation; 0.01% DMSO was kept as Vehicle for ATRA. One-way ANOVA statistical test was performed (B) Relative expression of AQP3 and AQP9 comparing expression levels between neutrophils and ATRA+G-CSF differentiated NB4 cells, T-student's test was performed (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

The AQP3 and AQP9 expression levels in NB4 cells differentiated with ATRA alone or in combination with G-CSF were evaluated by RT-qPCR.

The combined effect of ATRA+GCSF led to a significant increase in *AQP9* mRNA expression levels up to 12-fold when compared to the ATRA-differentiated cells, notably no changes were detected in expression levels of *AQP3* mRNA when the two conditions were compared (Fig. 24A).

Despite the up-regulation effect seen by the G-CSF, the AQP9 gene expression was still 100-fold less expressed when compared to the circulating neutrophils (Fig. 24B).

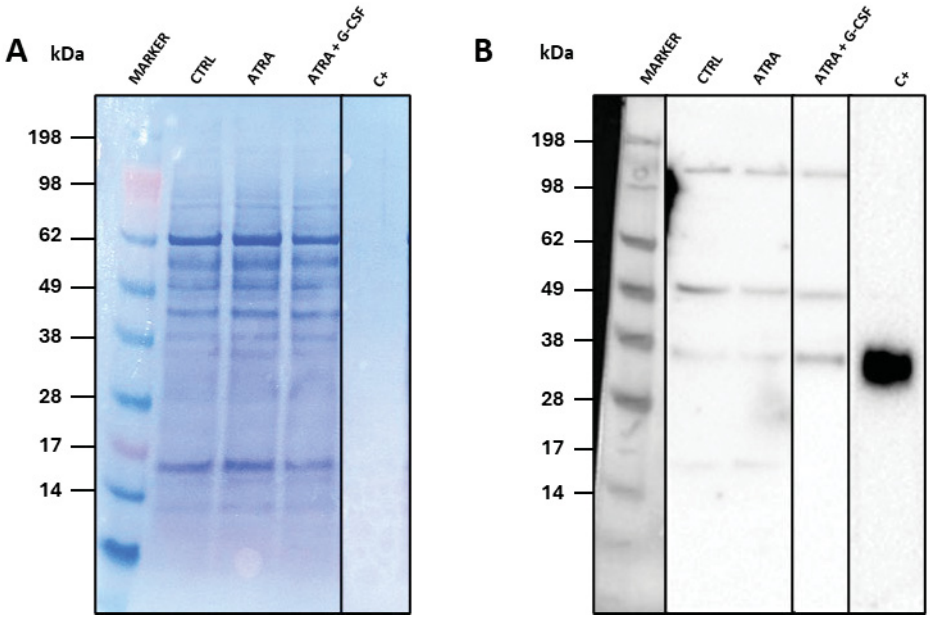


Figure 25 – Western blotting analysis of AQP9 in differentiated NB4 cells. (A) Coomassie-Blue stain of electron-transferred PVDF membrane. (B) Immunoblot reactivity of AQP9 in basal NB4 (C), ATRA-differentiated NB4 (A), ATRA+GCSF differentiated NB4 and positive-control represented by CHO cells transfected with the construct of human AQP9 under the control of Tetracycline inducible promotor.

Coherently with the RT-qPCR results, stronger immunoreactivity was seen via western-blotting in lysates from ATRA+G-CSF differentiated NB4 cells as a band of 33/35 kDa representing AQP9 monomer, coherent with the immunoreactive band visualized in lysates deriving from Chinese Hamster Ovary (CHO) cells overexpressing the human construct of AQP9 under the control of TET inducible promotor (Fig. 25).

Immunofluorescence analyses were also carried out to further confirm AQP9 expression and localization in ATRA+G-CSF differentiated NB4 cells, coherently with the RT-qPCR and Western blotting analysis. Stronger immunolabelling was seen on cell membrane of stimulated cells compared to unstimulated cells (Fig. 26).

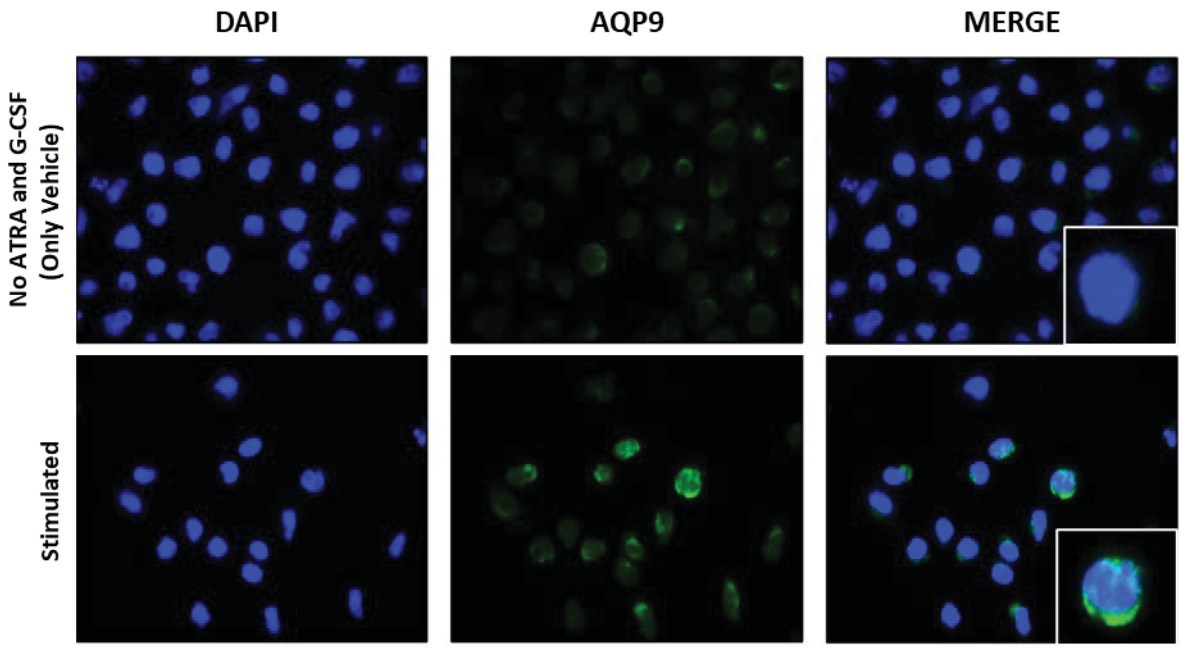


Figure 26 – **Immunofluorescence analysis of AQP9 in differentiated NB4 cells.** Differentiated cells were submitted to immunofluorescence analysis, nuclei were stained with DAPI in blue, AQP9 was stained with rabbit anti-AQP9 (#SAB4301752 Merck) in green, respectively the channels were merged. No ATRA and G-CSF cells have been treated for 5 days with 0.01% DMSO (Vehicle of ATRA) while Stimulated cells have been treated for 5 days with 1 μM ATRA + 10 ng/mL G-CSF.

II.3.5.2. Blockade of Aquaporin-9 alters the phospho-proteome profile of NB4 cells challenged with pro-inflammatory stimuli

In a preliminary study conducted in collaboration with Dr. Michael Rützler (CEO and co-founder of ApoGlyx AB), ATRA+GCSF differentiated NB4 cells were challenged with a mix of pro-inflammatory inductors (100 ng/mL LPS, 10 ng/mL TNFα, 10 ng/mL IL-6 and 100 nM fMLP) in presence or absence of the specific AQP9 inhibitor RG100204 (Mohammad et al. 2022), phosphorylation patterns in kinases were then assayed using the Human Phospho-Kinase Array kit (Biotechne, R&D Systems, Minneapolis, Minnesota, USA).

Table 7 – Summary table of the tested proteins by using the Proteome Profiler Human Phospho-Kinase Array

Proteins detected by the Proteome Profiler Human Phospho-Kinase Array (phosphorylation site)		
Akt 1/2/3 (S473)	HSP60	PRAS40 (T246)
Akt 1/2/3 (T308)	JNK 1/2/3 (T183/Y185, T221/Y223)	Pyk2 (Y402)
beta-Catenin	Lck (Y394)	RSK1/2 (S221/S227)
Chk-2 (T68)	Lyn (Y397)	RSK1/2/3 (S380/S386/S377)
c-Jun (S63)	MSK1/2 (S376/S360)	Src (Y419)
CREB (S133)	p38α (T180/Y182)	STAT1 (Y701)
EGFR (Y1086)	p53 (S15)	STAT2 (Y690)
eNOS (S1177)	p53 (S392)	STAT3 (S727)
ERK1/2 (T202/Y204, T185/Y187)	p53 (S46)	STAT3 (Y705)
Fgr (Y412)	p70S6K (T389)	STAT5a/b (Y699)
GSK-3 alpha/beta (S21/S9)	p70 S6 Kinase (T421/S424)	STAT6 (Y641)
GSK-3 beta (S9)	PDGFRβ (Y751)	WNK-1 (T60)
HSP27 (S78/S82)	PLCγ1 (Y783)	Yes (Y426)

By using this array, we were able to detect the effects of AQP9 gating, in challenged cells, on proteins involved in signaling pathways which regulate various immune cells functions such as: migration and/or adhesion (e.g. FGR, pDGFRβ, Src), inflammasome activation (e.g. STAT family and WNK-1) and stress response pathways (e.g. p53, RSK 1/2 and STAT1).

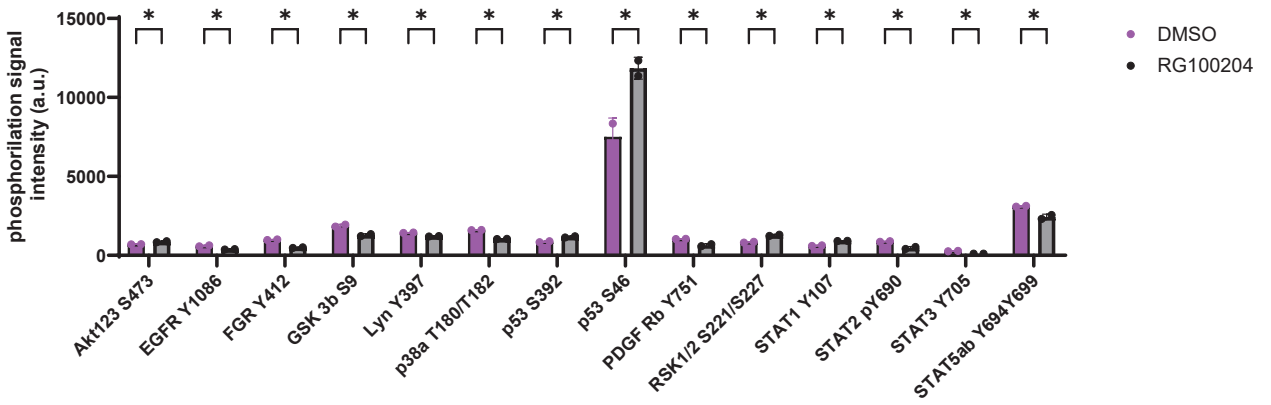


Figure 27 – Phosphorylation analysis of cell signaling pathways mediators on differentiated NB4 cells challenged with pro-inflammatory activators. Challenged NB4 cell lysates were assayed using the Human Phospho-kinase array kit, most of the NF-κB and TLR4 signaling pathway proteins assayed showed a significantly lower level of phosphorylation when cells were incubated with the specific inhibitor of AQP9, while the p53 protein showed a higher level of phosphorylation which could be related to a greater inhibition of NF-κB functionality exerted by the active form of the p53 protein. DMSO (0.5% v/v) was kept as vehicle control. *, $p < 0.05$, T-student's test was conducted between conditions.

The AQP9 inhibition led to a significant shift in phosphorylation patterns in several proteins (Fig. 27), starting from the p53 protein, typical checkpoint protein which phosphorylation activates apoptotic pathway cascade in mature Neutrophils upon ROS-related DNA damage, stimulated by bacterial infections (Garrison et al. 2010), highlighting the role of AQP9 during the redox homeostasis in Neutrophils.

Other changes in protein phosphorylation might reveal a role for AQP9 in modulating the activation of inflammatory-related signaling pathways, such as TOLL-like receptor signaling pathways and B-cell receptor signaling pathways, which modulate the activation of downstream pathways such as the NF-κB signaling pathway and the JAK-STAT pathways, modulating both the inflammatory cytokine production and the cell survival upon activation.

Altogether, the lower phosphorylation levels detected in FGR, PDGFRβ and STAT family proteins, which are key regulators in migration and adhesion related pathways suggest a potential role for AQP9 in modulating these immune cells functions upon stimulation, which will be furtherly assessed by *in vitro* assay.

II.3.6. DISCUSSION

Here, we evaluated the possibility of using the NB4 cell line as a cell platform for studying the functional involvement of AQP9 in human neutrophils.
Most of the studies have reported a role for Aquaporin-3 in NLRP3 inflammasome activation exploiting cell lines able to mimic Macrophages, like THP-1 cell line (da Silva et al. 2021), the most commonly used cell line to study macrophages functions and pathways.
During the last few years, neutrophils have gained a lot of attention, being the first defense line of the human body against invading pathogens. Unfortunately, most of the studies relied on animal models to study the effect of AQP9 gating and/or ablation to study inflammatory related pathways (da Silva et al. 2022; Mohammad et al. 2022; Tesse et al. 2021).
Animal models represent the gold-standard platform for studying immune system functions, but the knowledge transfer between mice and humans is always complex due to the diverse complexity involved in the two organisms.
Our work aimed at characterizing the acute promyelocytic leukemia (APL) cell line NB4 to mimic human circulating neutrophils to prove them as a suitable model to study AQP9 involvement in neutrophil related functions and associated pathways.
AQP9 increase of expression upon ATRA treatment in the NB4 cells was previously exploited and proposed as a therapeutical strategy to treat APL with Arsenic-Trioxide administration (ATO) in patients affected by leukemia to selectively target the cancerous cells (Iriyama et al. 2013).
The RT-qPCR results show significant up-regulation of AQP9 upon differentiation via combined effect of ATRA and G-CSF. This is confirmed by immunofluorescence and Western blotting associated to the flow cytometry analysis of the expression of the CD15 specific marker of mature neutrophils.
During differentiation, neutrophils undergo different metabolic shifts, here the AQP9 up-regulation might be relevant to maintain the homeostasis of differentiating committed cells, by using it as an escape route for metabolic by-product such as Lactates and ROS, which are synthesized after a shift from an aerobic glycolytic pathway to an increased mitochondrial respiration and Free-fatty acid metabolism (Kumar and Dikshit 2019).
Our preliminary analysis conducted on NB4 cells challenged with pro-inflammatory inductors (LPS, TNFα, IL-6, fMLP) revealed their ability to respond to noxious stimuli by regulating the activation of key modulators of Toll-like receptors signaling pathways through PI3K-Akt axis and JAK-STAT axis, capable of modulating the activity of NF-κB transcription factor.
The gating of AQP9 by using the isoform specific and potent inhibitor RG100204 led to an increase in p53 phosphorylation levels when compared to its vehicle control, p53 protein is a well-known transcription factor which induces the expression of genes involved in apoptosis, cell cycle arrest and DNA repair (Harms, Nozell, and Chen 2004; Liu and Chen 2006; Riley et al. 2008). In resting cells the p53 protein levels are maintained at low levels due to its interaction with its negative regulator Mdm2 (Harms, Nozell, and Chen 2004). Upon cell stress and/or DNA damage p53 is phosphorylated, disrupting the p53-Mdm2 complex, leading to p53 stabilization and increased transcriptional activity (Chene 2003).
Our results reveal that AQP9 gating leads to a significant alteration in the phosphorylation status of several proteins involved in essential cellular functions. Notably, we observed a decrease in the phosphorylation levels of many components of the NF-κB and TLR4 signaling pathways, which are cru-

cial for mediating inflammatory responses. The diminished phosphorylation of kinases such as FGR, PDGFRβ, and various STAT family members suggests that AQP9 plays an important role in modulating cell migration, adhesion, and overall inflammatory signaling (Mohammad et al. 2022; Thomas and Brugge 1997; Andrae, Gallini, and Betsholtz 2008; Levy and Darnell 2002; O'Shea et al. 2015). On the other hand, the increased phosphorylation of p53, a key checkpoint protein involved in the apoptotic response to oxidative stress, indicates that blocking AQP9 may enhance p53-mediated pathways. This shift hints a potential mechanism by which AQP9 influences redox homeostasis in neutrophils, thereby affecting their survival and functional response to inflammatory stimuli(Garrison et al. 2010) by enhancing the anti-inflammatory effect exerted by the phosphorylated p53 protein (Chene 2003; Liu and Chen 2006; Riley et al. 2008; Webster and Perkins 1999).

Further evaluations will be performed via migration and adhesion *in vitro* assays to investigate whether the reduced phosphorylation of these key regulators is associated with impaired cell motility or altered interactions with the extracellular matrix.

In summary, our findings suggest that AQP9 is a critical mediator of inflammatory and stress-response signaling in neutrophils. The observed alterations in phosphorylation patterns upon AQP9 inhibition underscores its potential as a therapeutic target for controlling inflammatory processes. Future study will focus on elucidating the molecular mechanisms by which AQP9 influences these pathways.

II.3.7. CONCLUSIONS

Our study provides evidence supporting the use of the NB4 cell line as a reliable platform for investigating the involvement of aquaporins in human neutrophil functions. Traditionally, research on Aquaporins in the context of inflammation has focused on AQP3 using macrophage-like models such as the THP-1 cell line (da Silva et al. 2021; da Silva and Soveral 2021), while studies on neutrophils have largely relied on animal models and/or murine derived neutrophils (da Silva et al. 2022; Mohammad et al. 2022; Tesse et al. 2021; Bromberger et al. 2022). Here our work aimed to fill this gap by characterizing NB4 cells as a model for human circulating neutrophils, with a particular focus on AQP9.

Our findings demonstrate that differentiation of NB4 cells with ATRA and G-CSF leads to a significant up-regulation of AQP9, as confirmed by RT-qPCR, immunofluorescence and Western blotting analysis, this up-regulation might be relevant for maintaining cell homeostasis during the metabolic shifts that occur in differentiating neutrophils, potentially serving as an escape route for metabolic by-products such as lactates and reactive oxygen species (Kumar and Dikshit 2019).

Phosphorylation profiling demonstrated that inhibiting AQP9 in differentiated NB4 cells substantially modulates critical signaling pathways (including the JAK-STAT axis and FGR-PDGFRβ mediators) thereby impacting cell migration and/or adhesion as well as inflammatory responses.

AQP9 might therefore be a key mediator of inflammatory and stress-response signaling in neutrophils. The alterations in phosphorylation patterns upon AQP9 inhibition not only affect pathways central to inflammatory signaling but also hint at potential impacts on cell migration and adhesion. Future in vitro assays will focus on these functional aspects to fully elucidate the consequences of AQP9 modulation.

III.1. GENERAL CONCLUSIONS

Sepsis and inflammatory disorders represent, nowadays, a subject of numerous studies with the immune cells category being the most investigated actors in inflammatory diseases.
To investigate the role of AQPs in sepsis as both prognostic and diagnostic biomarkers, as well as novel pharmacological target, we first investigated the expression and distribution of AQP3 and AQP9 in immune cells, such as PBMC and neutrophils isolated from human peripheral blood.
The first chapter established the differential expression of AQP3 and AQP9 in PBMC and neutrophils, highlighting their distinct roles in immune cell functionality, where AQP3 might be necessary for the activation of the NLRP3 inflammasome in Monocytes while AQP9 might be relevant for cell motility and extravasation of neutrophils. We then focused on septic patients to demonstrate the relevance of AQP3 and AQP9, respectively evaluated in PBMC and neutrophils, as diagnostic and prognostic biomarkers in sepsis. By integrating bio-statistical and machine-learning approaches, our study led to the designing and production of the first iteration of our ML-driven algorithm capable of providing sepsis diagnosis and 28-day mortality prognosis, shedding new lights on the possible role of AQPs as easily analyzable biomarkers for sepsis screening.
The relevance of AQPs as drug targets in sepsis using the NB4 cell line is object of the third chapter where the cells are evaluated as suitable platform to test the involvement of AQP9 in several neutrophil-related pathways, such as NLRP3 inflammasome priming and activation, cell migration and/or adhesion and cytokine release.
The results achieved in this doctoral thesis represent the first step toward understanding the intricate role of AQPs in inflammatory responses, thereby paving the way to studies aimed at proposing novel therapeutical strategies targeting AQPs.

III.2. FUTURE PERSPECTIVES

The findings of this doctoral work underscore the potential of AQP3 and AQP9 as novel biomarkers for sepsis prognosis. Nonetheless, further studies are essential, particularly through the expansion of patient cohorts to include a more heterogeneous population. This approach will enhance the sensitivity and robustness of our machine learning algorithm leading to its validation in real-world clinical settings, facilitating personalized treatment administration and improved patient stratification in cases of suspected infection.

Promising evidence from murine models, where AQP9 gating has emerged as a viable therapeutic strategy for polymicrobial sepsis (Tesse et al. 2021; Mohammad et al. 2022), we now plan extensive studies using the NB4 cells as a novel human-derived model to closely investigate the role of AQP9 in NLRP3 inflammasome activation, bridging the existing gap between animal studies and human-centered research in inflammatory contexts.

Moreover, the encouraging results obtained in the third chapter let us believe that AQP9 is deeply involved in regulating key functions of human neutrophils including: cell migration and/or adhesion; inflammatory and cell stresses responses. Future studies will exploit live-cell imaging and cytokine profiling to further elucidate the downstream effects of AQP9 modulation on neutrophil activity, deepening our understanding of its role in the immune system.

III.3. PUBLICATIONS AND CONGRESS PROCEEDINGS

Manuscripts in preparation:

Gena P, Garra S, Zagaria N, Mentino D, Calamita G. Aquaglyceroporins in liver energy metabolism: roles, regulation and translational relevance. *In preparation*.

Gena P, Mentino D, Garra S, Zagaria N, Mastrodonato M and Calamita G. Lack of Aquaporin-9 ameliorates the severe metabolic disorder and inflammation triggered in the murine model of Wolman disease. *In preparation.*

Garra S, Hymøller CM, Di Molfetta D, Zagaria N, Cardone RA, Rützler M, Birkelund S, Calamita G. Selective inhibition of AQP3 and AQP9 impairs human white blood cells migration. *In preparation.*

Zagaria N, Garra S, Gena P, Rützler M, Calamita G. Granulocytic maturation of promyelocytic NB4 cell line requires AQP9 but not AQP3. *In preparation*.

Selected oral communications and posters at congress and meetings:

- "Adipose and hepatic aquaglyceroporins in energy metabolism: physio-pathology and translational relevance" (poster 56ASM-0163 S6 – 56th ESCI Annual Scientific Meeting, Bari, 8-10 June 2022)
- "Aquaporin-3 is upregulated in the monocytes of patients with Familial Mediterranean Fever (FMF)" – **Selected for oral communication** (56ASM-0230 - 56th ESCI Annual Scientific Meeting, Bari, 8-10 June 2022)
- "Gene deletion of Aquaporin-9 normalizes oxidative stress and improves survival in a bacterial endotoxin induced mouse model of sepsis" (poster P1.6 – 72nd SIF National Congress, Bari, 14-16 September 2022)
- "Inhibition of AQP3 reduces hydrogen peroxide uptake and LPS-dependent redox signaling in human PBMC" – **Selected for oral communication** (poster PIII-8 – Benzon Symposium no. 66 Aquaporin 2022: Aquaporins in health and disease, Copenhagen, 26-29 September 2022)
- "White blood cells aquaporins AQP3 and AQP9 are implicated in NLRP3 inflammasome dysfunction underlying Familial Mediterranean Fever (FMF)" (poster P1.14 - 72nd SIF National Congress, Bari, 14-16 September 2022)
- "Aquaporin-9 (AQP9) is involved in the systemic inflammation of LPS-induced endotoxic shock in mouse" (Poster P-012 – European Phagocyte Workshop 2023, Budapest, 29 March – 1 April 2023)
- "Aquaporin-9 (AQP9) plays a role in the early phase of the systemic inflammation of a murine model of endotoxic shock" (poster P1.50 – 73rd SIF National Congress, Pisa, 06-08 September 2023)
- "Selective inhibition of AQP3 reduces the hydrogen peroxide uptakeand the LPS-dependent redox signaling and cell migration of humanperipheral blood mononuclear cells (PBMC)" (poster P1.17 - 73rd SIF National Congress, Pisa, 06-08 September 2023)

- “Ablation of Aquaporin-9 (AQP9) ameliorates the phenotype of a murine model of lysosomal acid lipase deficiency (Wolman disease)” (poster P2.13 – 73rd SIF National Congress, Pisa, 06-08 September 2023)
- “Selective inhibition of two aquaporin membrane channels, AQP3 or AQP9, impairs human PBMC and neutrophil cell migration” – **Selected for oral communication** (European Phagocyte Workshop 2024, Visegràd, 20-23 March 2024)
- “White blood cells AQP3 and AQP9 are involved in NLRP3 inflammasome activation and have relevance as prognostic biomarkers in sepsis” (poster P3.66 – 74th SIF National Congress, Roma, 11-13 September 2024)
- “Aquaporin-9 plays a role in the severe metabolic disorder and inflammation in a murine model of lysosomal acid lipase deficiency (LAL-D)” – **Selected for oral communication** (74th SIF National Congress, Roma, 11-13 September 2024)
- “Selective inhibition of two aquaporin membrane channels, AQP3 or AQP9, impairs human PBMC and neutrophil cell migration” (poster P2.40 - 74th SIF National Congress, Roma, 11-13 September 2024)
- "Aquaporins as novel biomarkers and potential therapeutic targets in sepsis: physiopatological and machine learning-driven approaches" – **Selected for oral communication** (Dottorati STEM: un’opportunità di sviluppo – Meeting – Bari, 5 November 2024)
- “Selective blockade of two aquaporin water channels, AQP3 and AQP9, impair human WBC migration” (submitted – European Phagocyte Workshop 2025, Madrid, 26-28 March 2025)

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