Aquaporins:

A Channel to Understanding the Pathogenesis of Chronic Rhinosinusitis

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Table of Contents

Thesis Summary

Aquaporins (AQPs) are cell membrane water transport channels and their discovery has revolutionised the understanding of water movement through tissue and tissue remodelling in the last 20 years. In chronic rhinosinusitis (CRS), there are pathological features suggestive of aberrant sinonasal water transport including altered composition of secretions, mucosal oedema, tissue remodelling and polyp formation. This project was undertaken to investigate a possible link between AQPs and CRS.

Chronic rhinosinusitis (CRS) is a chronic, inflammatory condition of the nose and paranasal sinuses that affects up to 10% of the Australian population. Characterised by inflammation of the sinonasal mucosa, CRS was defined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 (EPOS 2012) based on clinical symptoms and investigation findings. Chronic rhinosinusitis (CRS) is further classified phenotypically into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). However, AQP expression in normal and CRS sinus tissue remains incompletely understood. This study provides baseline knowledge of sinonasal mucosa AQP expression for the future investigation of AQPs in the pathogenesis of CRS. The hypothesis of this thesis was that AQP expression and location are altered in CRS in comparison to normal sinonasal mucosa.

Methods

Sinonasal tissue was collected during endoscopic sinus surgery or trans-sphenoidal surgery from three patient groups: normal controls, CRSwNP and CRSsNP. The mRNA expression of human AQP0–AQP12b was determined using quantitative real-time PCR. Cellular localisation of AQP1, AQP3, AQP4, AQP5, AQP7 and AQP11 was determined by immunohistochemistry.

Results

The mRNA of AQP0–AQP11 was identified in all samples; however, AQP12b mRNA was not detected. Statistically significant differences in the mRNA expression levels of AQP4 and AQP11 were identified between normal and CRSwNP patients ($p<0.05$). Differences in the cellular localisation of AQPs were observed in both CRSsNP and CRSwNP patients vs. normal controls. More intense localisation to the cell cytoplasm was observed for AQP5 in glandular epithelium

(CRSwNP; $p<0.05$) and surface epithelium (CRSsNP; $p<0.05$), and AQP4 in glandular epithelium (CRSsNP; $p<0.05$).

Conclusion

This study characterises normal human sinonasal AQP mRNA expression and protein localisation. The findings correlate well with the few published studies in this area, and extend the knowledge of AQP expression in human sinonasal tissue by providing normal baseline AQP expression profiles for future reference. Increased intracellular localisation of AQP4 and AQP5 was identified in both phenotypes of CRS, raising interesting questions regarding the significance of these findings in CRS aetiology. Future work will focus on the implication of intracellular AQPs on water flow and/or tissue remodelling in CRS.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that, to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference is made in the text.

Dr Claire Frauenfelder

Acknowledgements

Completion of this project has been a team effort and I gratefully acknowledge the assistance of my supervisors and mentors, Professor A.S. Carney, Dr Charmaine Woods, Dr Damian Hussey and Dr Eng Ooi.

Ethics and tissue selection

The ethics application was completed as part of a larger project commenced prior to my time in the department by Dr Charmaine Woods in conjunction with other members of Flinders ENT. Some samples used in this project were obtained from the Flinders ENT tissue bank and RNA had already been extracted. During the course of the project, I actively recruited patients to contribute tissue samples to this study, as well as the tissue bank. I personally identified suitable patients for inclusion and meticulously checked all clinical records (from both public and private practice case records), imaging and pathology to ensure that each participant was adherent to the inclusion and exclusion criteria.

PCR component of study

After training by Dr Woods, Dr Hussey and research assistants Tingting Wang and Alfiya Ansar in the Flinders University Upper Gastrointestinal (GI) Laboratory, I extracted RNA from newly acquired samples. I then performed all spectrophotometry, RNAase treatment, cDNA synthesis, real-time quantitative polymerase chain reaction (PCR) and verification of PCR products. An electronic workbook for recording experimental data in line with local laboratory protocols was kindly modified for this project with the assistance of Dr George Mayne. The data normalisation and analysis were performed with input from Dr Hussey and Dr Woods, with some training from Tingting Wang.

Immunohistochemistry component of study

The formalin-stored samples were processed and paraffin blocked at the time of collection by SA Pathology at a fee to the department. Immunohistochemistry (IHC) slides were prepared from the paraffin blocks by research assistant Kim Griggs from the Department of Anatomical Pathology for a fee. Anti-AQP antibodies 1, 3, 4 and 5 had previously been optimised by Ms Griggs during departmental investigations, and she supervised my acquisition of skills in tissue and slide preparation, IHC

labelling and antibody optimisation for the remaining AQPs. Microscopy training was provided by Yvette DeGraff as part of my orientation to the Flinders Microscopy facilities. The scoring system and skills in reading AQP-labelled slides were developed in conjunction with Associate Professor Sonja Klebe. Further assistance and training in slide reading and scoring were provided by Dr Woods (Flinders ENT), Kim Griggs and Dr David Astill (Department of Anatomical Pathology, Flinders Medical Centre). Statistical advice regarding the use of Likert scale data was sought during a consultation with Associate Professor Richard Woodman, School of Medicine, Flinders University.

Thesis proof-reading

The thesis was proof read by Valerie Williams (Professional Writing Services).

Additional contributors

To my friends, siblings and colleagues: thank you for your patience and support.
To my mother, Judy Frauenfelder; we have walked a hard road together: thank you.

Finally, completion of this thesis would not have been possible without the dedicated support of my husband, Dr Eamon Raith. You have been there for every up and the myriad of downs. Your encouragement, patience, love of academia and wise advice have kept me going and I am eternally grateful.

Publication arising from this thesis

Aquaporin expression profiles in normal sinonasal mucosa and chronic rhinosinusitis.

Frauenfelder C, Woods C, Hussey D, Ooi E, Klebe S, Carney AS.

International Forum of Allergy and Rhinology. November, 2014;4:901-908

Presentations arising from this thesis

2013 **Aquaporins: a channel to understanding chronic rhinosinusitis?**
American Rhinologic Society Annual Meeting, Vancouver, Canada

2013 **Altered sinonasal aquaporin mRNA expression in chronic rhinosinusitis – a new focus**
The Australian Society for Medical Research:
2013 South Australian Scientific Meeting

2012 **Water transport through sinonasal mucosa: a role for aquaporins in chronic rhinosinusitis?**
The Australian Society for Medical Research:
2012 South Australian Scientific Meeting

List of abbreviations

18S rRNA: 18S ribosomal ribonucleic acid
AQP(s): Aquaporin(s)
ASL: Airway surface liquid
ATP: Adenosine triphosphate
BBB: Blood-brain barrier
cDNA: Complementary DNA
CFTR: Cystic fibrosis transmembrane conductance regulator
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
CRS: Chronic rhinosinusitis
CRSsNP: Chronic rhinosinusitis without (sans) nasal polyps
CRSwNP: Chronic rhinosinusitis with nasal polyps
CT: Computed tomography
DNA: Deoxyribonucleic acid
ECM: Extracellular matrix
ER: Endoplasmic reticulum
FAK–MAPK: Focal adhesion kinase–mitogen-activated protein kinase pathway
FESS: Functional endoscopic sinus surgery
gDNA: Genomic deoxyribonucleic acid
H&E: Haematoxylin and eosin
HPRT: hypoxanthine phosphoribosyl transferase
IHC: Immunohistochemical
MIP: Major intrinsic protein of cell membrane
MMP: Matrix metalloproteinase
mRNA: Messenger
NPA motif: Asparagine-proline-alanine motif
PCL: Periciliary layer
PCR: Polymerase chain reaction
P_f: Tissue water permeability
PKA: Protein kinase
PKC: Protein kinase C
PI3K/Akt: Phosphoinositide 3-kinase/protein kinase B
qRT-PCR: Quantitative real-time polymerase chain reaction
RNA: Ribonucleic acid
RS: Rhinosinusitis
RT: Reverse transcription
SCC: Squamous cell carcinoma
TIMP: Tissue inhibitors of metalloproteinases
UPW: Ultra-pure water

Literature Review

Chapter 1. Aquaporins (AQPs)

1. 1 Introduction

Aquaporins (AQPs) are a family of intrinsic cell membrane proteins that facilitate water movement across cell membranes in many organisms.[1, 2] The study of AQPs has exploded rapidly since they were identified as cell membrane water transport channels in the 1990s, with some aspects of AQP study very detailed, while in others basic questions remain unanswered.[3, 4]

Thirteen (13) mammalian AQPs have been discovered.[5, 6] They share some common water transport properties but research continues into additional functions.[7] To date, studies have investigated AQP expression and function in the lower respiratory tract; however, AQP expression and function in human sinonasal tissue and chronic rhinosinusitis (CRS) have not been comprehensively examined.

1. 2 AQP homology and the MIP family

The major intrinsic protein (MIP) family is a large family of cell membrane transport channel proteins expressed in cell membranes.[6] The AQPs are a subfamily of the MIP family, and these mammalian proteins are classified by structural homology as AQP1 (Figure 1.1).[8] After the discovery of AQP1, all known cell membrane proteins were reviewed for the expression of a characteristic asparagine-proline-alanine (NPA) motif (see Section 1.3: AQP protein structure) and those identified were renamed AQPs according to the Human Genome Nomenclature Workshop.[9] As new proteins with the NPA motif were discovered, they have been added to the AQP family.[6] The overall homology between AQP proteins is in the order of 25–40% but, in the area around the characteristic NPA motif, this is much higher.[10, 11]

Figure 1.1: Aquaporin family tree

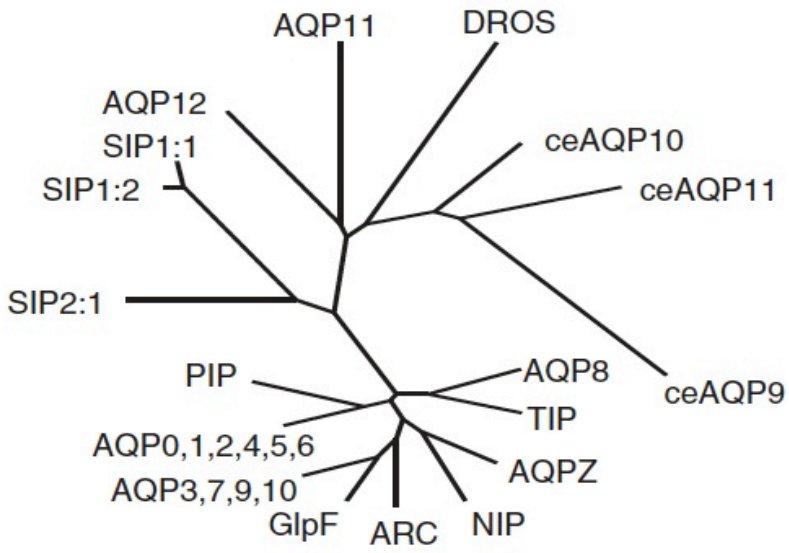

Figure 1.1: This figure demonstrates the AQP family tree. It shows the subgrouping of the most recently discovered "superaquaporins" AQP11 and AQP12, which have a significantly different structure when compared to the existing human AQP sub-types. Figure 1.1 also includes closely related plant, insect and bacterial MIPs that are structurally similar to mammalian AQPs (DROS, SIPs, PIP, GlpF, ARC, NIP, TIP, AQPZ and ceAQPs). The image is reproduced with permission from Ishibashi 2008.[6]

There are three subfamilies of mammalian AQPs: (1) aquaporins that transport water molecules only (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8);
(2) aquaglyceroporins that transport water and also small molecules such as glycerol, urea and carbon dioxide (AQP3, AQP7, AQP9 and AQP10); and (3) the most recently discovered "superaquaporins" (AQP11, AQP12a and 12b) which have the NPA motif but are less than 20% homologous to other mammalian AQPs.[4, 6, 12] The prefix "super" refers to the fact that the primary structure of these proteins shares little similarity to other AQPs, and that the proteins should be classified as part of the AQP "superfamily" in terms of structural classification. Investigations are continuing into their function.[6]

1. 3 AQP protein structure

1. 3. 1 Primary structure

Mammalian AQPs are polypeptides between 28000–37000 Da in size and are encoded on a range of chromosomes (Table 1.1).[13]

Table 1.1: Aquaporin protein characteristics

Aquaporin	Gene Location	Size	Amino Acids	Family
MIP (AQP0)	12q13.3	28122 Da	263	Aquaporin
AQP1	7p14.3	28526 Da	269	Aquaporin
AQP2	12q13.12	28837 Da	271	Aquaporin
AQP3	9p13.3	31544 Da	292	Aquaglyceroporin
AQP4	18q11.2	34830 Da	323	Aquaporin
AQP5	12q13.12	28292 Da	265	Aquaporin
AQP6	12q13.12	29370 Da	282	Aquaporin
AQP7	9p13.3	37232 Da	342	Aquaglyceroporin
AQP8	16p12.1	27381 Da	261	Aquaporin
AQP9	15q21.3	31431 Da	295	Aquaglyceroporin
AQP10	1q21.3	31763 Da	301	Aquaglyceroporin
AQP11	11q14.1	30203 Da	271	Superaquaporin
AQP12a	2q37.3	31475 Da	295	Superaquaporin
AQP12b	2q37.3	31475 Da	295	Superaquaporin

Table 1.1: Key protein characteristics for each AQP are listed in the table. Information is sourced from Safran 2012.[13]

The AQP primary structure incorporates characteristic NPA motifs. Located at sequence positions 76–78 and 192–194, the asparagine-proline-alanine NPA motifs have revealed an apparent duplication of amino acids within the AQP.[14] The N and C termini halves of the protein are almost symmetrical (Figure 1.2).[1, 2, 15] Both the N and C termini are on the cytoplasmic surface of the cell membrane.[16]

Figure 1.2: Schematic diagram of AQP4 primary sequence

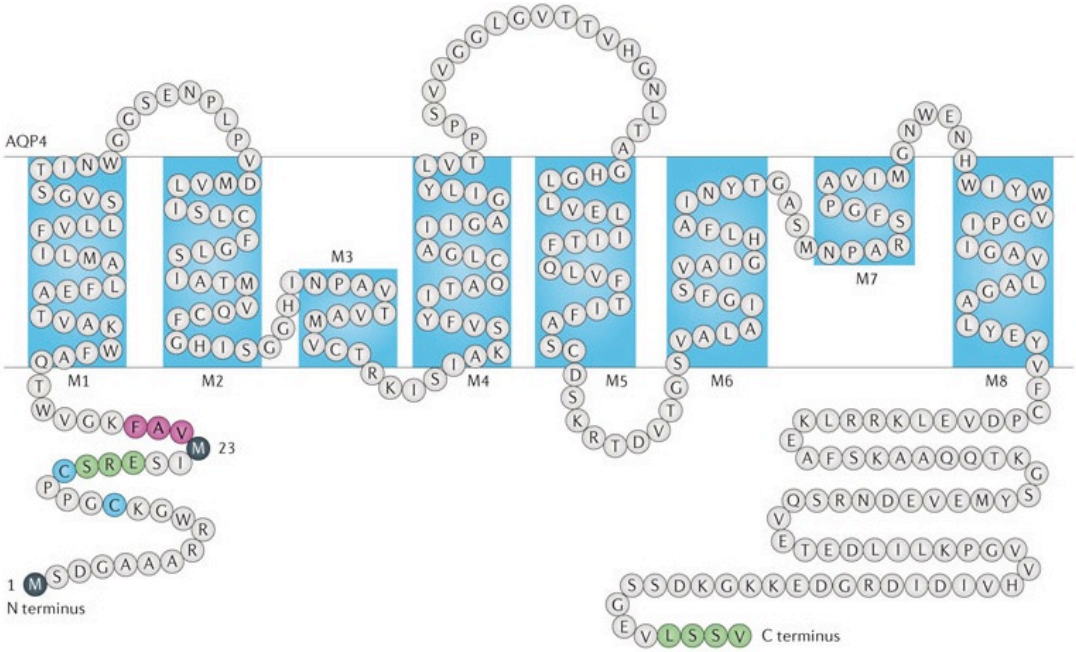

Figure 1.2: This diagram demonstrates the N and C termini of the AQP4 protein on the intracellular surface of the cell membrane. The image is reproduced with permission from Papadopoulos and Verkman.[17]

1. 3. 2 Secondary structure

The AQP subunit (monomer) is made up of six alpha-helix domains 1–6, each of which spans the cellular bi-layer membrane, and they are connected by five loops (A–E) (Figure 1.3).[16, 18] Loops B and E are longer than the others and contain the functionally significant NPA motifs, which are key to the tertiary structure despite their distance from each other in the primary sequence.[14]

Figure 1.3: Schematic diagram of AQP monomer prior to folding

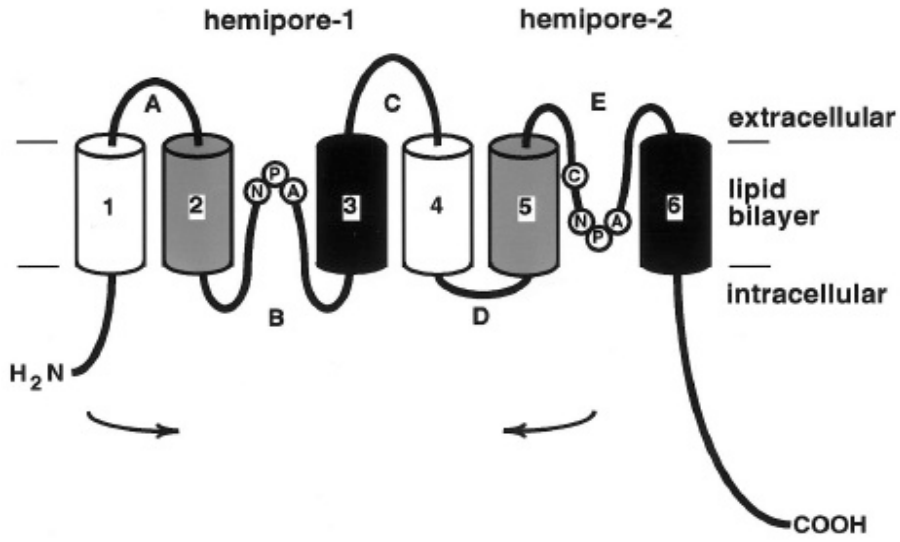

Figure 1.3: This figure demonstrates an unfolded AQP monomer with six alpha-helical domains crossing the cell membrane. The NPA motifs are seen on loops B and E. These two areas become adjacent when the AQP folds in its tertiary structure and create the central water-channel pore (see Figure 1.4). The image is reproduced with permission from Borgnia et al.[18]

1. 3. 3 Tertiary structure

AQP monomers have a distinctive three-dimensional shape that facilitates extremely efficient water transport at $3x10^9$ water molecules per second.[19] This is the key property of the AQP family and the result of the AQP tertiary structure (the water transport is described in detail in Section 1.6: AQP monomer pore.[18, 20] The unique folding process of an AQP protein creates a central pore (Figure 1.4). The transmembrane domains 1, 2, 4 and 5 fold allowing the hydrophobic NPA motif of cytoplasmic loop B to associate with the extracellular NPA motif on loop E. Next, van der Waals forces act between the polar residues Asn-76 and Asn-192 (one from each NPA motif) and attract the two areas. These areas meet in the middle of the cell membrane, forming the hourglass-shaped conducting central pore in the AQP monomer.[1, 11, 19]

Figure 1.4: Characteristic AQP central hourglass pore formed by NPA motifs

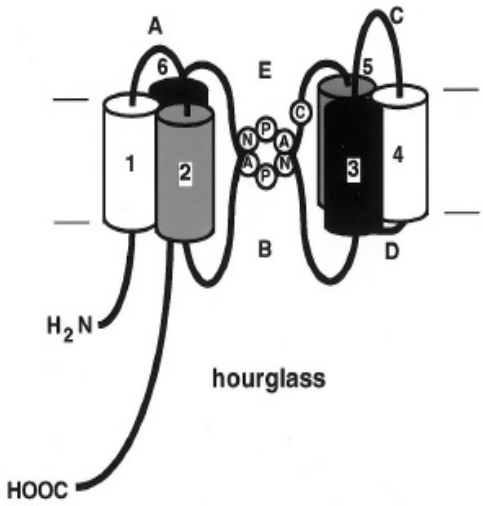

Figure 1.4: This is a schematic diagram of the tertiary structure of an AQP. The intrinsic cell membrane protein's folding is demonstrated with the characteristic NPA motif forming a central pore through which water molecules move. The image is reproduced with permission from Borgnia et al.[18]

The central pore acts as a water channel and sometimes transports other uncharged molecules, depending on the individual AQP primary sequence and central pore diameter.[10] The structure of each mammalian AQP protein varies slightly, resulting in unique central pore transport properties and giving rise to the subfamilies: aquaporins, aquaglyceroporins and superaquaporins.[21]

1. 3. 4 Quaternary structure

The quaternary structure of the AQP is a tetramer (Figure 1.5).[11] AQPs are stable as a monomer and still functionally able to transport water through each monomer's central pore. However, in nature, all AQPs are expressed only as homotetramers in cell membranes (i.e. units made up of four identical monomer subunits).[10, 11, 22-26] The functional significance of this tetrameric configuration remains unclear. Multiple domain sites interact between adjacent monomers, with hydrogen bonds forming between them within the AQP tetramer.[11, 19] It is hypothesised that a symmetrical homotetramer makes a more stable membranous constituent but no formal evidence for this exists.[12] Shi et al. created artificial heterotetremers by inserting three synthetic and non-functioning monomers into an AQP tetramer with a single functional monomer.[27] The single functional monomer operated independently as a normal water channel, indicating ongoing and independent function of each AQP monomer despite their usual expression as homotetramers.

Figure 1.5: Tetrameric structure of AQP1

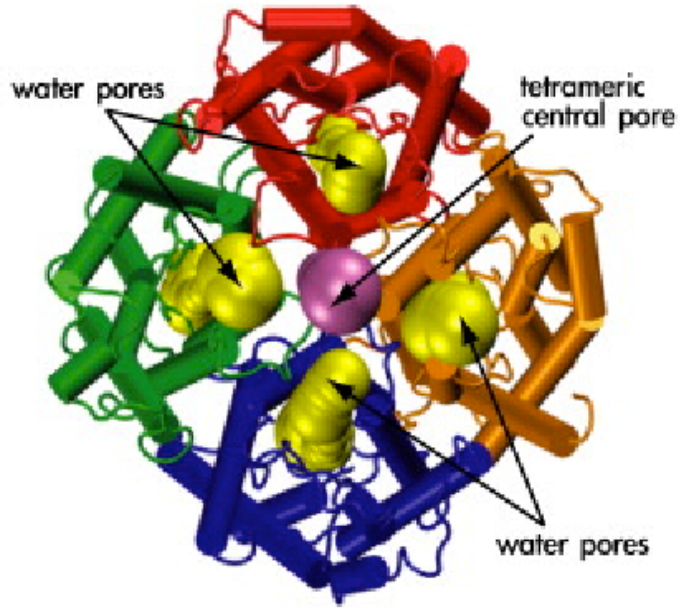

Figure 1.5: This diagram shows four AQP monomers held together by interactions between multiple sites along each transcellular alpha-helical domain. The view is from above the tetramer with the four peripheral water pores (central to the individual monomer, yellow) in addition to the larger central tetrameric pore (pink). The image is reproduced with permission from Yu et al.[25]

The large, central AQP tetramer pore is water and proton impermeable; however, its function remains an unresolved mystery.[11, 25] One group has hypothesised that the central pore acts as an ion transport channel when activated by cyclic guanosine monophosphate (cGMP).[23, 24, 26] Yool et al. demonstrated that ion transport via the tetramer pore is more than just leakage through the larger diameter pore or some other passive mechanism of pore activation[26]; however, these findings are not widely accepted.[28] In experimental conditions, AQP1 was shown to act as a gated ion channel, opening after activation by cGMP to conduct sodium.[25] Early work by Yasui et al. also demonstrated anion conduction by AQP6 in very low pH experimental conditions.[29]

Once expressed in the cell membrane, AQPs tend to form large arrays, for example, AQP0 in the lens of the eye and AQP4 in renal tissue.[30] The volume of AQP proteins expressed in a region of the cell membrane is proportional to water permeability in that cell membrane area.[5, 20] The physiological role remains unexplained of this affinity of large numbers of tetramers to congregate.

1. 4 AQP protein biogenesis

Newly synthesised AQP protein is inserted into the endoplasmic reticulum (ER) membrane.[31] Upon insertion, it undergoes a conformational change to align appropriately. It then awaits trafficking in the vesicles to its final functional destination – usually the cell membrane.[32]

When AQP1 is inserted into the ER membrane, a conformational change in the tertiary structure of the monomer results in a 180-degree rotation of the C-terminus part of the protein.[33] During AQP production, only four of the AQP domains (1,3,5 and 6) cross the ER membrane. However, after complete insertion of the AQP into the membrane, structural rotation occurs (i.e. a change in tertiary structure) and all six domains are aligned in a transmembrane fashion.[34, 35] The AQP1 and AQP4 undergo this process, but studies on the other AQPs have yet to be published.[32]

1. 5 Regulation of AQP function

The AQP water transport requirements are not uniform across tissue types and AQP function is regulated by different mechanisms in different tissues.[11, 12] Some AQP regulatory mechanisms have been established; however, work in this area continues.[11, 36]

1. 5. 1 Hormonal regulation

Hormone-regulated AQP trafficking has been most extensively studied in renal collecting ducts, where AQP2 is key in determining urine concentration (Figure 1.6).[37] Vasopressin is the hormone released by the posterior pituitary in response to body dehydration. It binds at the basolateral membrane of renal collecting duct cells, triggering cyclic adenosine monophosphate (cAMP) synthesis and protein kinase A activation. The AQP2 contained in intracellular vesicles is phosphorylated, and the vesicles are transported to the cell apex. Fusion of the vesicles incorporates AQP2 into the apical membrane, resulting in increased AQP2-mediated reabsorption of water by the collecting duct epithelium, concentrating urine and increasing overall body water. Little evidence exists for hormone changes in CRS; however, other circulating cell signalling molecules (e.g. inflammatory cytokines or growth factors) may act at the sinonasal mucosa and alter AQP regulation in CRS.

Figure 1.6: AQP2 trafficking in renal duct collecting cells

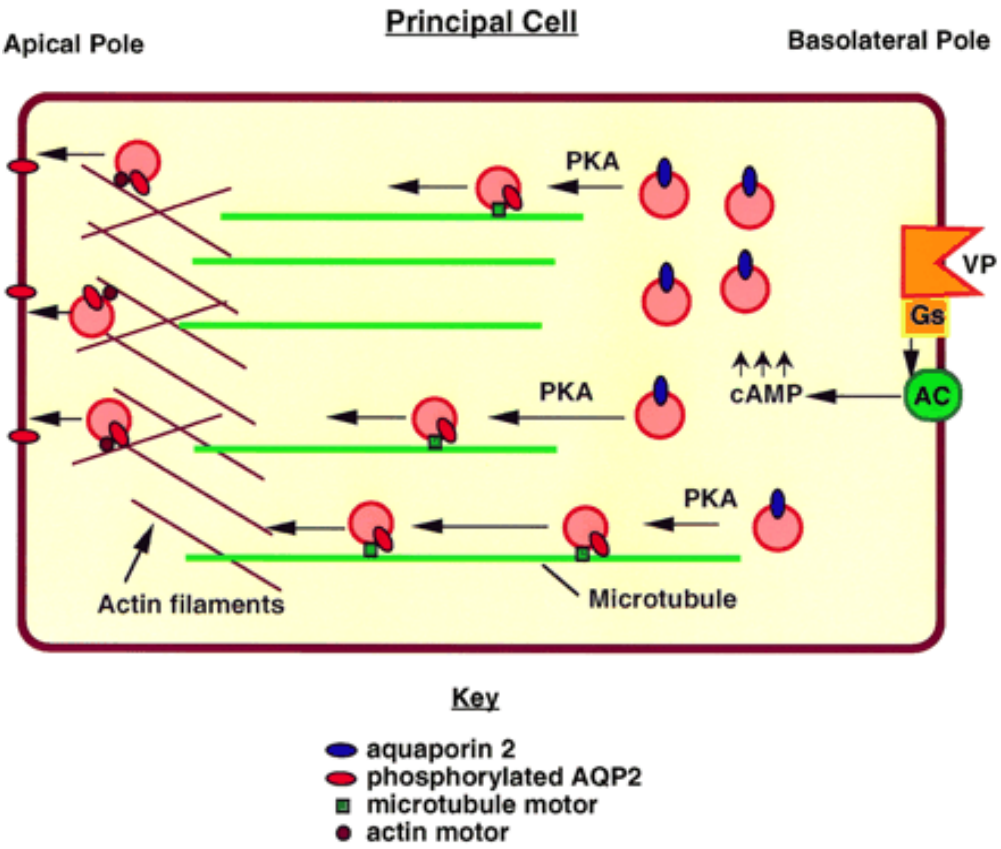

Figure 1.6: This diagram shows the action of vasopressin at the basolateral membrane of renal collecting cells which results in AQP2 insertion at the cellular apex. The up-regulation of the AQP2 which is inserted into the apical membrane results in increased reabsorption of water by the collecting duct epithelium. The image is reproduced with permission from Brown [37]

1. 5. 2 Phosphorylation

Phosphorylation activation sites within AQP protein structures have been identified.[11] AQP1 is regulated by protein kinase C (PKC) activation in response to an osmotic water gradient in serosal surfaces such as the pleura or the peritoneum. AQP1 expression is stimulated by phosphorylation of sites Thr(157) and Thr(239) on PKC, resulting in increased cell membrane permeability.[38] The role of phosphorylation in AQP2 trafficking has been previously described above. As knowledge of AQP phosphorylation improves, other sites of AQP activation or inhibition may be identified, and may become therapeutic targets.

1. 5. 3 Ionic and pH regulation

Regulation of AQPs by ionic compounds is an area of ongoing research.[39] Mercurial ions and other heavy metals inhibit several AQPs.[1, 18, 29, 40-44]

The pH also stimulates different permeability in different AQPs.[41] Hydrogen ions bind to AQP proteins at the histidine sites in loops A and C, regulating the water permeability of the central monomer pore. AQP0 was more permeable in an acidic pH, while transport by AQP4 increased in alkaline pH.[41] The AQP3 pore closes in an acidic environment[45], while AQP6 was shown to be not functional until the pH was lowered below 5.5.[29]

1. 6 AQP monomer pore: water channel function and molecular selectivity

The key function of all 13 mammalian AQPs is water transport regulation. The AQP water transport is novel as it is bi-directional, responds to osmotic or hydraulic gradients, and there is no resultant membrane current from the water flow.[18, 46] The AQP monomer pore has three functional regions: the extracellular vestibule; the charged pore constriction (midway between the cell membrane lipid bi-layers); and the cytoplasmic vestibule.[46]

The pore size and charge at the central pore constriction site determine the rate of water flow by the AQP.[10, 19] A smaller pore diameter correlates to more tightly regulated water movement. The diameter of AQP1 is 2.8 angströms, only slightly larger than a single water molecule, and allows strict exclusion of all molecules larger than water.[10] The pore constriction arises from tertiary folding of the AQP protein. The amino acid expressed at location AA182 (close to the NPA motif) varies between AQPs, and is responsible for pore constriction and alteration of the pore charge (Table 1.2).[10, 26, 46, 47] Water transport was inhibited when mutations were inserted around the NPA motif protein sequence, demonstrating the pivotal role this area plays in pore water permeability.

Table 1.2: Comparison of AQP water permeability characteristics

AQP	Water permeability	AQP	Water permeability
AQP0	Water (low)	**AQP7**	Water (high)
AQP1	Water (high)	**AQP8**	Water (high)
AQP2	Water (high)	**AQP9**	Water (low)
AQP3	Water (high)	**AQP10**	Water (low)
AQP4	Water (high)	**AQP11**	Water (low)
AQP5	Water (high)	**AQP12**	Water (low)
AQP6	Water (low)		

Table 1.2: This table shows that different AQPs have different water permeability characteristics. Table 1.2 is reproduced with data from King et al. and Ishibashi[6, 10]

In water transport by means of other open transcellular membrane channels (such as gramicidin A), water molecules travel in a single file, forming strong hydrogen bonds between H_2O molecules.[48] This process carries additional protons across the cell membrane and has higher energy requirements than AQP-mediated transport.[19] The AQP pore constriction is a positive electrostatic field due to charged dipoles generated by protein folding of loops B and E (carriers of the NPA motifs) (Figure 1.7).[19]

Figure 1.7: Water molecule's transport via central pore of AQP monomer

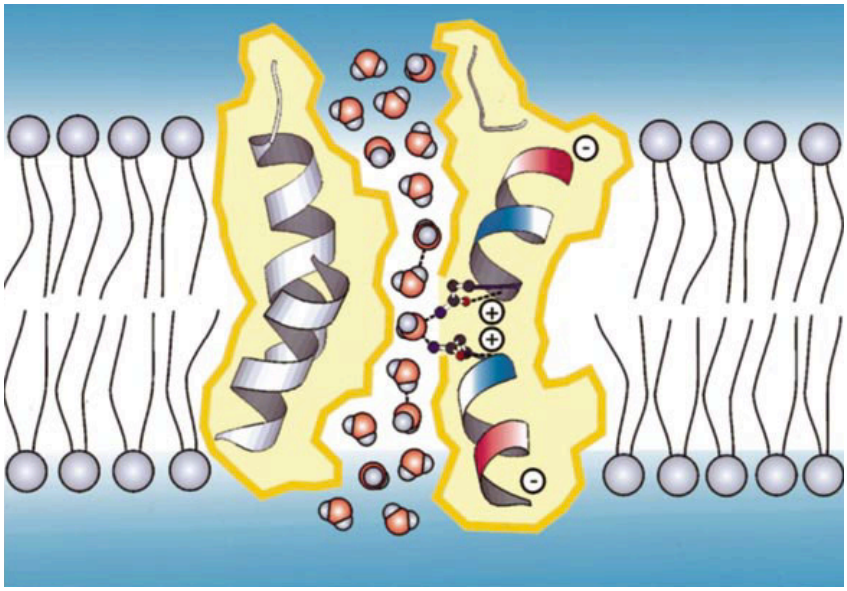

Figure 1.7: This diagram demonstrates how, after the AQP protein folds, the orientation of the two halves of an alpha-helix are dipoles pointing toward the central pore and the resultant polar charge restricts passage of all (including water) molecules through the site. The image is reproduced with permission from Murata et al.[19]

Water passes through the pore constriction in single file; however, unlike other channels, the AQP constriction re-orientates the H_2O hydrogen ions and prevents hydrogen bonds forming between them. The water molecules enter the vestibule where the pore walls are lined with specific hydrophobic molecules that act as a funnel.[10, 49] This streamlined transport method is low-energy, efficient and replicated rapidly, transporting up to $3x10^9$ water molecules through each monomer per second.[1, 19]

The variation in pore constriction between AQPs allows some AQPs to transport non-polarised solutes, metalloids, gases and other neutral solutes.[50] Further work in this area will clarify the regulation of the transport of these other molecules.

1. 7 Additional features and functionality of mammalian AQPs

The study of AQP roles beyond that of water transport is diverse: investigators have demonstrated that AQPs have more complex functions than as simple water channels (Figure 1.8).[7, 39, 51] Associations between AQPs and other functions continue to be identified; however, precise mechanisms of many of these interactions are yet to be

established.[7, 32] Examples of additional AQP functions are described and illustrated below on Figure 1.8.

Figure 1.8: Functions of human AQPs throughout the body

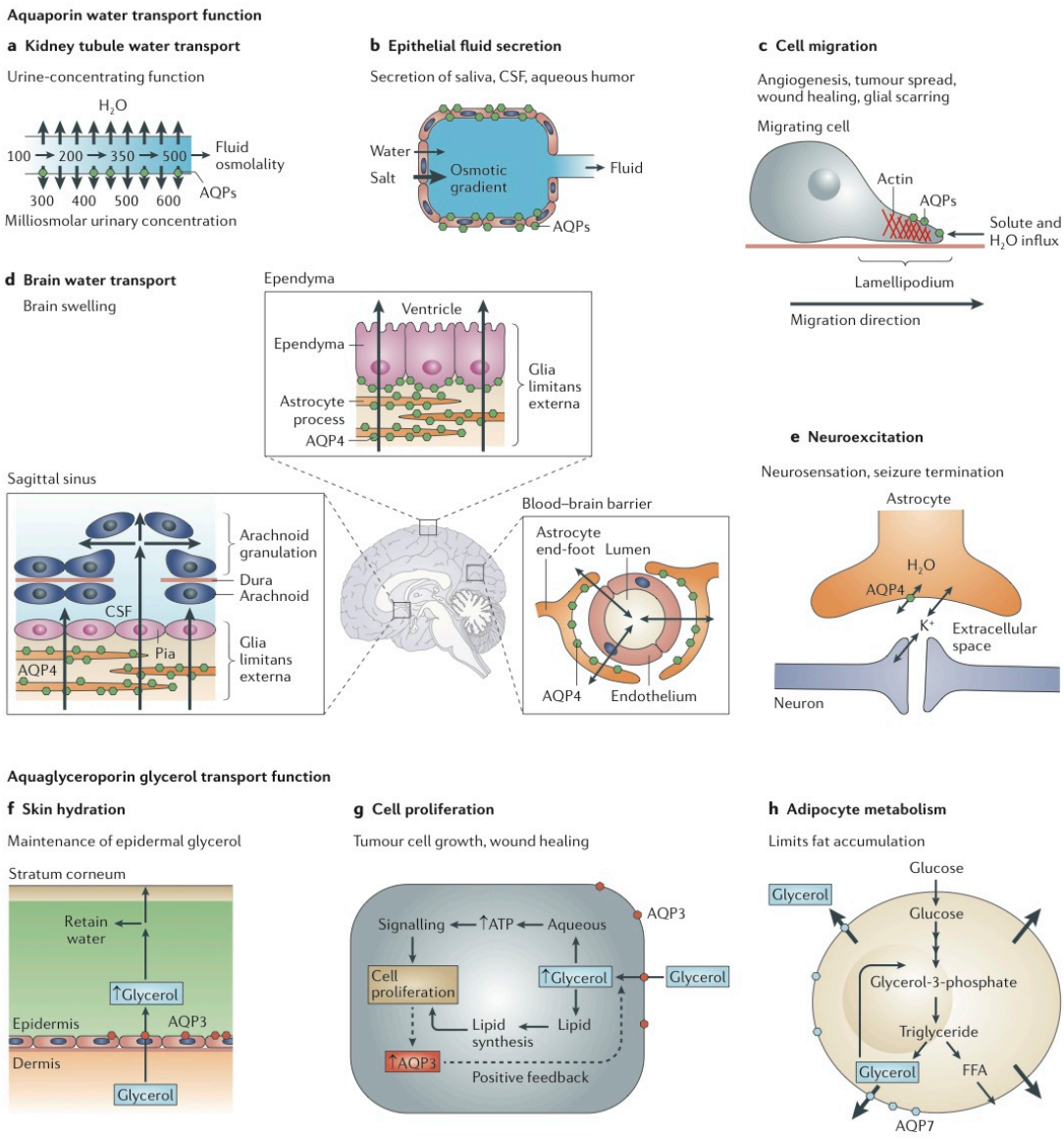

Figure 1.8: This diagram schematically presents a range of AQP functions in many tissue types throughout the body. The image is reproduced with permission from Verkman et al.[39]

1. 7. 1 Tissue remodelling – cell migration, angiogenesis and wound healing

Tissue remodelling has been linked to AQPs in both normal tissue and disease by several mechanisms. Saadoun et al. studied the role of AQP1 and AQP4 in wound closure.[52] No changes in cell proliferation or cell adhesion were seen; however, cells expressing AQP1 and AQP4 migrated more rapidly to facilitate wound closure

(measured by the movement of the cells across a filter). The cells that migrated more quickly had a higher number of cellular protrusions and these protrusions turned over at a faster rate, accelerating migration. Lamellipodia and cell membrane ruffles (types of cellular protrusions) form at the leading edge of migrating cells (Figure 1.9). The protrusions arise after rapid local ion and water transport across the cell membrane which alters the cell volume.[53, 54]

Figure 1.9: AQP1 expression in cell migration

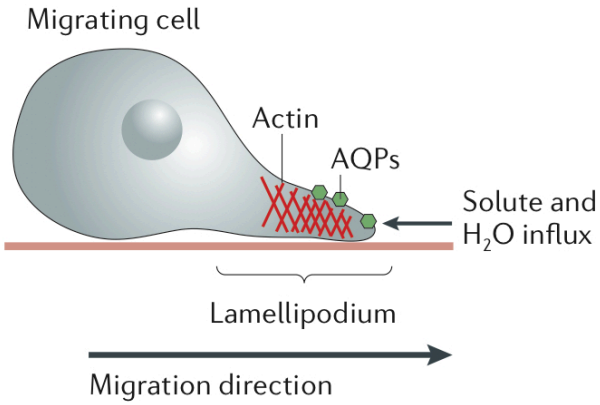

Figure 1.9: This diagram shows the mechanism for AQP1 localisation facilitating the influx of water during migration. AQP1 allows the water influx at the leading edge of the cell, increasing cell volume and facilitating cell protrusion (Lamellipodia) turnover. Cell migration is faster when there are more protrusions and they turn over rapidly. The image is reproduced with permission from Verkman et al.[39]

AQP1 has also been implicated in angiogenesis.[52] Blood vessel formation was impaired in AQP1-null mice, while tumours in AQP1-null mice had poor blood supply and were more necrotic. There was no difference in tumour growth in AQP3-null mice.

AQP3 is key to epidermal proliferation and cell migration in skin healing.[55] AQP3 has an established role in skin hydration and elasticity via the transport of glycerol[56], and the epithelial growth factor induces expression in human skin fibroblast migration during normal wound healing.[57] Delayed skin wound healing occurred in AQP3-null mice, while hyper-proliferative wound edges were seen when AQP3 expression was increased, suggesting a direct correlation between AQP3 expression and the rate of wound healing.[55]

1. 7. 2 Intercellular tight junctions

Another role of AQPs is in maintaining the integrity of intercellular junctions. AQP proteins on the surface of two adjoining cells form a tight configuration between the cell membranes creating an intercellular transfer channel capable of water transfer.[58] This has been observed in the eye where AQP0 forms homo-octomers: two AQP0 homotetramers joined to form an eight subunit entity. These units form tight cell junctions vital to maintaining lens hydration in the eye and vision, as water content dictates the transparency of the lens.[58]

1. 7. 3 Intracellular AQPs

Intracellular AQP expression is relatively uncommon in mammals when compared to plants.[59] Some AQPs have been identified in intracellular vesicles and within the membranes of intracellular organelles. AQP2 has been localised within intracellular vesicles in renal cells, awaiting signalling by vasopressin at the basolateral surface of the cell (described in Section 1.5: Regulation of AQP function).[37] AQP6 is a predominantly intracellular AQP, localised to intracellular vesicles in the renal tract; however, its function is less clear.[40] AQP8 was identified within hepatic and renal cells.[60]

The most recently discovered superaquaporins AQP11 and AQP12 were also localised within the cell.[61] AQP11 was localised inside hepatocytes, in brain neurons, and in renal proximal tubule cells—mostly likely at the membrane of the endoplasmic reticulum (ER).[62, 63] AQP12 was localised in pancreatic acinar cells. Study is continuing into the functional significance of intracellular AQPs.

Nozaki et al. have proposed the existence of intracellular water transport and have hypothesised that AQPs play a role in this process.[59] Water in the cell was previously thought to move passively along osmotic gradients; however, recent work proposes that there is free water (osmotically inactive) and bound water (involved in key organelle function, principally in mitochondria and at the ER). Mitochondria are primarily the principal site of aerobic cellular adenosine triphosphate (ATP) synthesis; however, they also have roles in cell signalling, growth, differentiation and cell death. Flux in mitochondrial volume correlates to the rate of oxidation as required substrates move in and out of the organelle, and it is hypothesised that AQPs contribute to this process.[59, 64]

1. 8 AQP expression and localisation

AQPs are found throughout the body and play a wide range of roles in tissue homeostasis.[5, 51]

1. 8. 1 AQP1

AQP1 was the first AQP identified and is the archetypal aquaporin.[3, 5] It was discovered incidentally on the surface of red blood cells during investigation of blood groups.[3, 65] Initially named "channel-forming integral protein of 28 kDa" or "CHIP28", the term "aquaporin" was designated by the Human Genome Nomenclature Workshop as additional members of the water channel family were identified.[9]

AQP1 is an aquaporin (i.e. transports water only) and is widely expressed throughout the body.[5] It is found in vasculature endothelium of non-fenestrated capillaries and venules, as well as in lymphatic vessels, and is expressed at the apical and basolateral surfaces of the endothelial cells.[66, 67] AQP1 has also been located in many epithelia in the body in, for example: the renal proximal tubule and loop of Henle (plays a key role in urinary concentration)[68]; pancreatic bile duct and acinar cells (contributing to bile secretion in fluid transport and within secretory granules)[5]; cerebral neurons and the choroid plexus (on the cerebrospinal fluid [CSF] luminal surface)[17]; in the eye (in the cornea, lens and vasculature)[69]; the ear [70]; and the respiratory tract.[67] Regulation of AQP1 function involves protein kinase C (PKC) and protein kinase PKA; however, the overall mechanism is unclear.[38, 71]

1. 8. 2 AQP0 (MIP)

Originally identified as the "major intrinsic protein of 26 kDa" or "MIP26", AQP0 was renamed after its homology to AQP1 was discovered.[5] AQP0 is a pure aquaporin and maintains important intercellular tight junctions. AQP0 has only been localised in the lens of the eye[58], and it is regulated by extracellular pH, calcium and zinc ions.[41, 42]

1. 8. 3 AQP2

AQP2 is an aquaporin and is abundantly expressed in the renal collecting duct.[72, 73] AQP2 has also been identified in the cochlea at the border of Reissner's membrane; however, studies were not able to prove a role for AQP2 dysfunction in Meniere's

disease.[74] The action of AQP2 in the renal collecting duct is regulated by vasopression (see Section 1.5: Regulation of AQP function).[37]

1. 8. 4 AQP3

AQP3 is an aquaglyceroporin and was previously identified as glycerol intrinsic protein (GLIP).[5, 75] It is important to epidermal cell migration and is abundantly expressed at epithelial surfaces throughout the body.[55, 76] It has been localised to the renal tract, digestive system, the eye, skin and the respiratory tract (discussed in further detail in Section 3.1: AQPs in the respiratory tract).[5] Localisation of AQP3 varies between different epithelia; however, it is most commonly expressed at the basolateral surfaces.[5] The action of AQP3 is regulated by pH, nickel and copper ions.[43-45]

1. 8. 5 AQP4

AQP4 is an aquaporin important for neurological tissue homeostasis. It is abundantly expressed in the brain, spinal cord and neurons.[17] AQP4 expression is important in blood-brain barrier (BBB) permeability; however, AQP4-null mice are not grossly phenotypically different from wild type mice, indicating other contributors to BBB water transport. Experiments have indicated AQP4 is vital in dissipating pathological brain oedema: AQP4-null mouse disease models exhibited significantly worsened vasogenic brain oedema than those expressing AQP4 (including brain tumour, abscess, haemorrhage and refractory epileptic seizures).[17]

AQP4 was also localised to renal collecting ducts, skeletal muscle, the eye, vestibular apparatus of the inner ear and the respiratory tract (discussed in Section 3.1: AQPs in the respiratory tract). Duchenne and Becker muscular dystrophy patients were found to have depleted muscle AQP4 expression, and studies of AQP4-null mice identified some changes in fast-twitch muscle action.[77, 78] AQP4 permeability increases in alkaline pH; however, other regulatory factors have not been identified.[41]

1. 8. 6 AQP5

AQP5 is an aquaporin and contributes to secretory function throughout the body.[5] AQP5 is abundantly expressed in the upper respiratory tract and in type I pneumocytes (discussed further in Section 3.1.2)[79-83] AQP5 has also been localised in salivary glands, lacrimal glands, the cochlea, the cornea and glands within the stomach.[5, 80, 84, 85] Regulation of AQP5 action is influenced by cAMP via the PKA

pathway[86], with some work currently investigating a biphasic response to cAMP and the beta-adrenergic agonist terbutaline.[87]

1. 8. 7 AQP6

AQP6 is an aquaporin and almost exclusively intracellular.[29, 40, 88] AQP6, while not the subject of many studies, was localised to intracellular membranes of renal cells.[40] AQP6 mRNA is present in mouse cerebellar tissue, and at tight junctions of rat parotid salivary glands.[89, 90] No studies have elucidated the functional significance of these findings. The permeability of AQP6 was regulated by changes in intracellular pH, and by mercurial ions.[88, 91]

1. 8. 8 AQP7

AQP7 is an aquaglyceroporin and is also known as AQPap (adipose).[5, 92] AQP7 was localised in tails of healthy, fertile sperm, while sperm lacking AQP7 had lower motility.[93, 94] It plays a role in small molecule absorption during digestion, and fat metabolism.[7, 95, 96] AQP7 also transports arsenite, a toxin and known carcinogen.[96, 97] This discovery is important to gain a better understanding of carcinogenesis and of the use of arsenite as a chemotherapeutic agent. AQP7 is regulated by insulin and vasopressin.[92, 98, 99]

1. 8. 9 AQP8

AQP8 is an aquaporin and is predominantly intracellular.[5] It is expressed within myoepithelial cells of many secretory tissues including the pancreas, salivary and airway glands, as well as in the gut, liver and kidney.[5, 60] AQP8 was also identified as a key transporter of ammonia into mitochondria, with ongoing investigation occurring into its role in other intracellular organelle functions.[64, 100] Experimental regulation of AQP8 by mercury and copper ions has been demonstrated.[101]

1. 8. 10 AQP9

AQP9 is an aquaglyceroporin known to also transport urea.[18] It has been localised in leukocytes (including neutrophils), the gut, liver, spleen and lung.[5] Phosphorylation of AQP9 mediates neutrophil volume and migration[102] and hepatocyte AQP9 expression is dependent on the nutritional state, transporting both glycerol and urea in response to metabolic activity.[103] Mucus-secreting gastrointestinal goblet cells express AQP9 at the basolateral surface; however, the functional significance of this has yet to be confirmed.[104] Insulin and leptin regulate AQP9 activity in fat and liver cells via the phosphatidylinositol 3-

kinase/Akt/mammalian target of the rapamycin pathway [105], while a recent study disproved earlier hypotheses that PKC regulates AQP9 function.[106]

1. 8. 11 AQP10

AQP10 is a recently identified aquaglyceroporin.[6] mRNA studies have identified coding for AQP10 in a variety of tissues; however, AQP10 protein has only been localised to the apical membrane of small intestine villi [107, 108] and adipocytes.[109] It has been hypothesised that AQP10 plays a role in the absorption of water and key small molecules from the gut lumen; however, functional studies have yet to be completed. AQP10 activity in adipocytes was regulated by insulin.[109]

1. 8. 12 AQP11

AQP11 is a superaquaporin.[6, 21] AQP11 is understood to be predominantly intracellular and has been localised to the liver, kidneys and brain, with only limited investigation to date of any functional significance.[61] AQP11-null mice developed a fatal polycystic kidney disease.[62] Within weeks of being born, the mice developed large cysts in the renal cortex thought to arise from the ER, but the cyst-formation mechanism is unclear.[61] AQP11 does not appear to play a role in water transport and its regulation is unclear at present.

1. 8. 13 AQP12

AQP12 is a superaquaporin and has been localised to pancreatic acinar cells.[6, 61] AQP12-null mice were more susceptible to severe pancreatitis in a chemically-induced disease model.[110] Regulation of AQP12 has not yet been studied.

1. 9 AQPs in pathophysiology

The alteration of AQP expression or localisation has only been implicated in the pathophysiology of a few diseases to date.[1, 4, 111] Pathological mechanisms associated with AQPs in disease include: altered transport of water (e.g. cerebral oedema, Sjogren's syndrome); altered localisation of AQPs (insertion at incorrect location, failure to be transported to functional area, e.g. nephrogenic diabetes incipidus); and altered cell cycle regulation due to AQP binding or signalling abnormality (e.g. roles in cancer).[4] AQP knockout models have been used extensively to establish AQP function in normal tissues, and to screen for physiological dysfunction when AQPs are absent.

1. 9. 1 Cerebral oedema

AQP4 plays an important role in water transport across the blood-brain barrier (BBB).[17] It has been implicated in cerebral oedema sub-types via different mechanisms.[112] It is unclear if altered AQP4 expression in cerebral oedema is causative or a result of physiological compensation in relation to oedema.[17]

Cytotoxic cerebral oedema occurs within brain parenchyma and the BBB remains intact (e.g. focal cerebral ischemia, spinal cord compression or water intoxication).[112] AQP4-null mice were used to establish AQP4 as the rate-limiter of water movement from cerebral vasculature into the brain, in response to cytotoxic cerebral oedema.[113, 114]

Vasogenic cerebral oedema occurs when the blood-brain barrier (BBB) is disrupted (e.g. by brain tumours, abscesses or subarachnoid haemorrhage).[112] Water and solutes are driven into brain tissue from the bloodstream via hydrostatic force, a process independent of AQP4. Rapid water transport is required to eliminate extra volume from the brain. In AQP4-null mice, the vasogenic oedema is unable to drain, implicating AQP4 in the physiological elimination of oedema in this setting.[17]

Another study investigating AQP4 in glioblastoma found significant and chaotic redistribution of AQP4 protein throughout the glioma tissue when compared to the normal, strictly localised position in the BBB (Figure 1.10).[115] AQP4 was expressed in a site at which water transport would not have been across the BBB or appear to have any other functional role; it is hypothesised that the change in AQP localisation contributes to the localised oedema. This example of the altered localisation of AQP protein indicates a mechanism that may apply in causing or perpetuating sinonasal mucosal oedema in CRS.

Figure 1.10: Altered AQP4 expression in glioblastoma

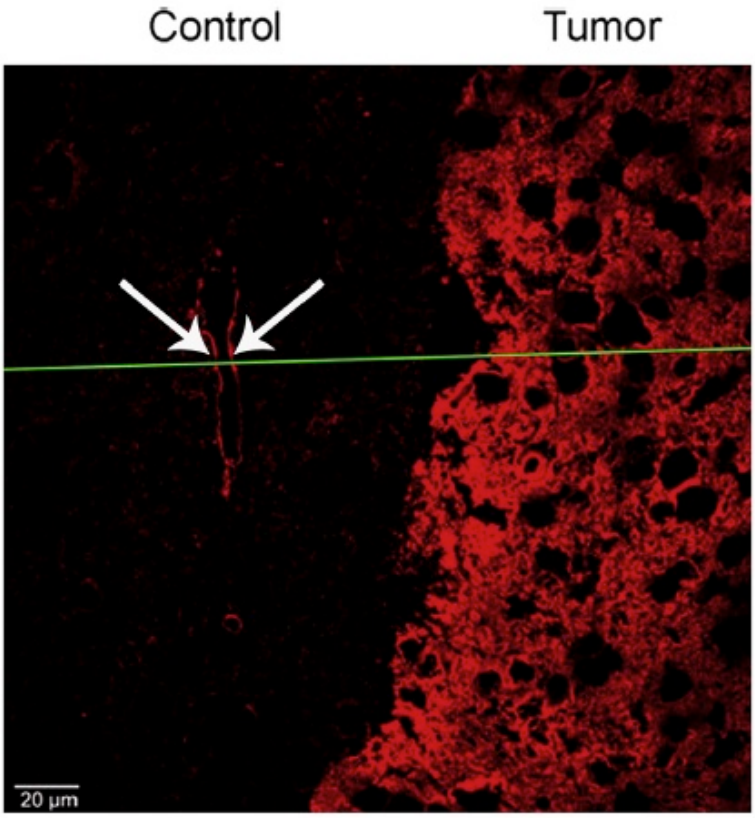

Figure 1.10: This image is of immunofluorescence of normal brain tissue and of glioblastoma. It demonstrates: (left) the specific localisation of normal AQP4 expression around the perivascular endfeet membrane, and (right) chaotic redistribution of AQP4 in glioma tissue. The image is reproduced with permission from Wolburg et al.[115]

1. 9. 2 Sjogren's syndrome

Sjogren's syndrome is an autoimmune disease that affects secretory glands and is characterised by thickened, dehydrated secretions[116] similar to those seen in CRS. In a normal salivary gland, the acini secrete isotonic fluid into the gland lumen from which large volumes of sodium chloride are then resorbed during the saliva's passage through the ducts.[117] AQP5 has been identified as the cell membrane protein critical in the tonicity of salivary secretions, as well as those produced by many gland types, including lacrimal glands.[118-120] In AQP5-null mice, increased tonicity and decreased salivary flow rate were demonstrated in multiple studies, without any other measure of global dehydration present in the rest of the mice's bodies.[119, 121]

Several studies of Sjogren's syndrome have identified altered AQP localisation, affecting secretory function within salivary and lacrimal glands.[80, 121-123] In salivary and lacrimal gland biopsies of patients with Sjogren's syndrome, AQP5 was localised to the glandular epithelial basement membrane, not to its usual apical position.[80, 124] Other studies of Sjogren's syndrome did not identify change in AQP3 and AQP5; however, they did find decreased AQP1 expression in glandular myoepithelial cells.[122, 123] This suggests a failure of protein trafficking or altered AQP5 protein polarisation in Sjogren's syndrome.[116] Given the similarities in thickened, dehydrated secretions between Sjogren's syndrome and CRS, it is possible that altered AQP expression or localisation contribute to the secretion properties that affect patients with these conditions.

1. 9. 3 Nephrogenic diabetes incipidus

AQP2 mutation disrupts normal urinary concentrating mechanisms, and causes a sub-type of nephrogenic diabetes incipidus.[4] Studies of AQP2 knockout mice identified defective urinary concentration, and subsequent investigation of patients with nephrogenic diabetes incipidus identified a sub-group who had an AQP2 mutation.[125, 126] The mutant AQP2 proteins were not incorporated at the collecting duct apical cell membrane, despite signalling by vasopressin at the basolateral membrane. The proteins were retained in the intracellular Golgi apparatus and were non-functional. It is possible in CRS that, even if AQPs are present (possibly detected at normal levels), there is a similar functional disturbance. To identify such an alteration in the proteins, not only would AQP expression profiles need to be established, but study of AQP function in sinonasal tissue would need to be undertaken.

1. 9. 4 Cancer

Altered AQP expression has been observed in cancers, affecting tumour growth as well as cell migration and metastases.[127]

In a gastric cancer study, Huang et al. observed increased AQP5 expression in the more proliferative tumours and those with enhanced lymph node metastases, while AQP5 inhibition slowed the malignant cell growth and metastasis.[128] In a non-small cell lung cancer study, AQP5 expression promoted local tumour invasion by altering phosphorylation at Ser156.[129] Downstream changes after phosphorylation induced malignant cell invasion into normal lung tissue. Higher AQP5 expression in the

patient tumour samples correlated with higher rates of recurrence and shorter disease-free survival periods.

AQP1 has been identified as an independent prognostic factor in malignant mesothelioma, with higher AQP1 expression in tumour tissue correlated to significantly increased survival time independent of a range of other disease and patient factors.[130] AQP1 is widely expressed in microvasculature, and has also been implicated in tumour angiogenesis and cell migration (as discussed in Section 1.7.1).[52, 54]

AQP3 expression is increased in primary oesophageal and lingual squamous cell carcinomas, and is associated with nodal disease when compared to normal local tissue.[131] An experimental AQP3 blockade inhibited the adhesion and growth of squamous cell carcinoma (SCC) cells via the focal adhesion kinase–mitogen-activated protein kinase (FAK–MAPK) pathway, achieved by decreasing phosphorylation of FAK. AQP3 was blocked with copper sulphate (a non-specific pan-AQP blocker) and AQP3 siRNA (small interfering RNA specific for AQP3). When Kusayama et al. combined AQP3 siRNA with cisplatin (a chemotherapy agent), there was apoptosis of SCC cells but not adenocarcinoma or fibroblast cells, indicating that AQP3 may be a therapeutic target in SCC.[131]

While CRS does not involve malignant transformation of sinonasal mucosa, the improved understanding of AQPs in malignancy and altered tissue remodelling may provide information applicable to the histological changes observed in CRS.

In gastric cancer, AQP3 has been found to positively regulate the activity of matrix metalloproteinases (MMPs), a key group of endopeptidases that regulate tissue remodelling, migration, angiogenesis, tumour cell invasion and metastasis.[132] However, in AQP3-null tissue, there was decreased phosphorylation of Ser473 in Akt (key to the PI3K/Akt intracellular signalling pathway), resulting in decreased expression of MT1-MMP, MMP-2 and MMP-9. While these findings are clearly relevant in future cancer research, MMPs have also been associated with tissue remodelling in CRS (as discussed in Section 4.2.4). A correlation between AQP and MMPs' activity indicates a future research area for CRS.

1. 10 AQPs as therapeutic targets

Few commercially available medications have been found to act on AQPs. Corticosteroids are a mainstay of treatment in CRS and some studies have begun to investigate the interaction between steroids and AQPs. Corticosteroids have been found to increase AQP1 expression in lung vasculature.[133-135] They also increase AQP1 expression in inner ear mucosa in a dose-dependent manner [136] and in peritoneal vasculature.[137, 138] In a human epithelial cell culture experiment, Tanaka et al. were able to induce AQP3 expression using a corticosteroid, but their work was unable to identify the specific mechanism for the change.[139] Up-regulation of the AQP3 promoter activity was observed; however, the location coding for the promoter was not able to be found and will require further study. In a mouse model of asthma, AQP1 and AQP5 were up-regulated after dexamethasone treatment.[140] In a final example, AQP5 expression decreased in nasal polyp tissue after treatment with intranasal corticosteroid.[141] No other literature investigating the link between AQP expression and corticosteroids was identified. To date, the mechanism for the response of CRS to corticosteroid treatment remains unexplained. It is possible that altered expression of AQPs in response to corticosteroids may increase reabsorption of water across sinonasal vasculature, decreasing localised tissue oedema, or decreasing sinonasal tissue hydration and resulting in less water availability for secretion production. Further work in this area would greatly increase our understanding of not only CRS, but also of the mechanism of the action of corticosteroids.

Heavy metals reversibly inhibit the action of AQPs; however, as treatment agents, metals are highly toxic to humans and animals.[142] Mercury, silver and gold inhibit AQP1 as well as plant AQP targets.[142-144] Nickel inhibits AQP3 reversibly and rapidly, but is not active against AQP4 or AQP5.[43] The applicability of these agents in human treatment remains unidentified. AQP1 is inhibited by carbonic anhydrase inhibitors, a group of anti-epileptic medications (e.g. acetazolamide and topiramate).[145-148] Finally, AQP-specific blockers are not yet available, limiting treatment options and slowing research developments; however, some newly developed compounds, such as tetraethylammonium (TEA+, a non-selective tetraalkylammonium ion channel blocker) have begun to appear in the literature.[39, 142, 149]

Chapter 2. The nose and sinuses

Key anatomical and physiological features of the nose and sinuses are next discussed, including specific discussion on respiratory epithelium, and an introduction to respiratory epithelium water transport, secretion and airway surface liquid production.

2. 1 Structure of upper respiratory tract

The nasal cavity is lined with stratified squamous epithelium at the nostrils, and covered by respiratory-type pseudostratified columnar epithelium elsewhere. The nasal turbinates are bony projections into the nasal cavities from the lateral and superior walls, covered in nasal mucosa continuous with the nasal cavity.[150] The four paired, air-filled paranasal sinuses (maxillary, ethmoid, sphenoid and frontal) are lined with respiratory-type mucosa continuous with the nasal mucosa. Located in the middle meatus is the osteomeatal unit (OMU), consisting of intricately folded mucosa and bone, which is the junction of frontal, anterior ethmoid and maxillary sinus drainage pathways.[151] Ciliated epithelial cells sweep the mucus produced in the sinuses into the nasal cavity along well-defined mucus drainage pathways.[151]

2. 1. 1 Nasal vasculature

The nasal cavity has specialised vasculature supplied from several arteries: the sphenopalatine and anterior ethmoid arteries postero-superiorly, the facial artery (via the superior labial artery) anteriorly, and the greater palatine artery inferiorly. The specialised sinusoidal venous plexus consists of abundant arteriovenous anastomoses, sinusoids and endothelial fenestrations that facilitate a high flow of heat and water across the epithelium, conditioning the air.[151, 152] Engorgement of the mucosa and nasal congestion results from physical activity as well as from reflexes to neural, thermal, psychological and chemical triggers, and shares characteristics with erectile tissue.[151, 153] The specialised vasculature is predominantly located on the inferior turbinate, as part of the middle turbinate and the septum.[154]

2. 1. 2 Sinonasal lymphatic drainage

Sinonasal mucosa contains superficial and deep lymphatics with many anastomoses to the dense local vasculature.[155] Lymph vessels are more densely concentrated in the paranasal sinuses than in the nose. Sinonasal lymphatics drain to the

retropharyngeal, submandibular and deep cervical nodes, and then to the parapharyngeal lymph nodes.[156]

2. 1. 3 Innervation

Nasal cavity sensation is supplied by the trigeminal nerve (ophthalmic and maxillary divisions) which communicates to the spinal trigeminal nucleus, thalamus and somatosensory cortex.[156] Vasomotor control of sinonasal mucosa (vasculature and nasal secretory glands) is parasympathetic, with some sympathetic input in secretion.[152, 157]

2. 2 Function of upper respiratory tract

The nose humidifies inhaled air before it passes through the nasopharnyx, larynx and trachea and enters the lungs.[150] Air temperature and humidification are regulated to optimise gas exchange and protect the sensitive lower airway.[158] Under normal conditions, the nose filters approximately 10,000 litres of air per day, and up to 30% of heat and moisture is reabsorbed in the nasal cavity on expiration.[159] The nasal mucosa shunts blood within the vasculature from region to region throughout the day causing cyclical engorgement, or the "nasal cycle".[151, 154] A constant overall airflow rate and mucosal surface area are maintained throughout the cycle which is under sympathetic control, via the vidian nerve.[155] The relevant neurotransmitters are noradrenaline and neuropeptide Y.[152] The opposing parasympathetic control of vasodilation is regulated by acetylcholine and vasoactive intestinal peptide (VIP). This blood flow ultimately hydrates the sinonasal mucosa and is the fluid reservoir for airway secretions.

The innate and adaptive defence mechanisms of the nose target inhaled pathogens.[153, 160] The nasal vestibule hairs filter large pathogens and airway surface liquid (ASL) traps almost 100% of the smaller ones (down to 4 μm in size).[161] The sinonasal surface epithelium is a physical barrier, blocking mucosal invasion by pathogens, and mediating the innate and adaptive immune responses.[162] Secreted ASL and the pathogens trapped in it are removed by mucociliary clearance.[163] Key immune peptides are secreted into ASL, including inflammatory mediators, lysozyme and other antimicrobial peptides.[162] Resident immune cells are scant in sinonasal epithelium, with some T cells present but almost no B cells in normal tissue.[155]

2. 3 Sinonasal epithelium

The sinonasal surface epithelium is respiratory-type pseudostratified ciliated columnar epithelium and originates from ectoderm, unlike the histologically identical lower respiratory tract which arises from endoderm.[164] External facial skin (stratified squamous epithelium) extends into the nasal vestibule and transitions to non-ciliated columnar epithelium. This anterior nasal epithelium merges with sinonasal pseudostratified ciliated columnar epithelium which lines the nasal cavity, nasopharynx and sinuses.[117, 165] The paranasal sinus epithelium is continuous with nasal epithelium with some minor differences: fewer seromucous glands and thinner, less vascular mucosa.[165]

The specialised olfactory epithelium is located in the nasal apex and upper septum.[165] In this area, there are additional olfactory nerve cells, modified microvilli on columnar cells and mucus-secreting Bowman's olfactory glands. For the purpose of this review, olfaction and olfactory mucosa will not be discussed further.

There are three main sinonasal epithelial cells: ciliated columnar cells, goblet cells and basal cells (Figure 2.1).[165] Approximately 70% of epithelial cells are ciliated columnar cells with microvilli (or cilia) at the apical surface. The cilia sweep in a coordinated fashion to move the ASL along drainage pathways to the nasopharynx. The cilia move in a whipping action with fast, propulsive force forward and a slower recovery stroke to resume their starting position. They will not function without: moist conditions, a minimum ASL depth of 10–15 μm and a temperature of 30°C, and are sensitive to environmental toxins, for example, cigarette smoke.[155]

Goblet cells comprise 15% of the sinonasal epithelium. They secrete mucus by exocytosis of mucin granules from the apical surface.[166] Basal cells anchor columnar cells to the basement membrane. Highly regulated tight junctions are present between the luminal epithelial cells, providing defence against pathogens and tightly regulating water flow.[167] Below the nasal epithelium, there are submucosal glands, sinonasal vasculature, nerves and connective tissue.

Figure 2.11: Sinonasal epithelium

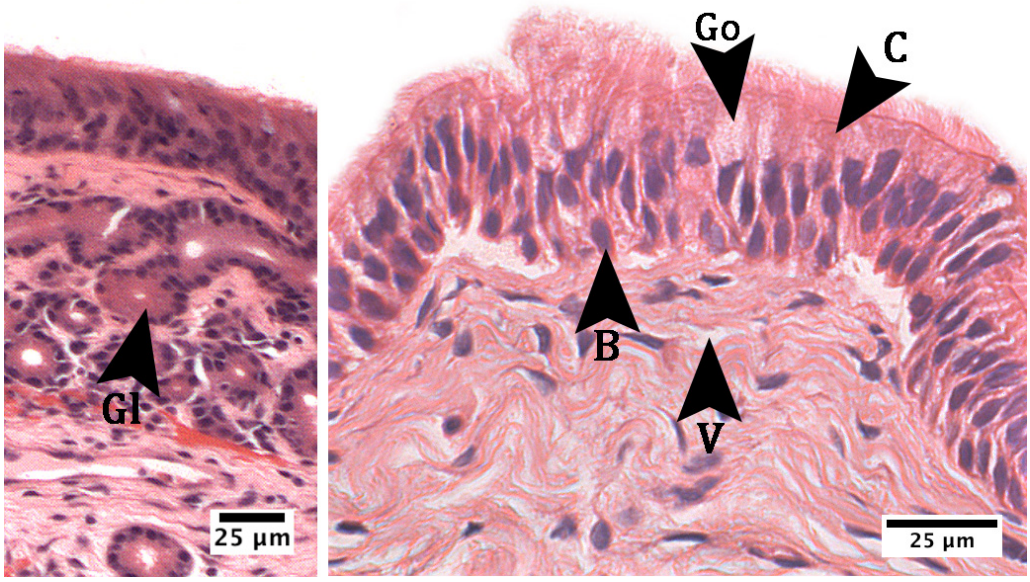

Figure 2.1: The above photomicrographs are normal sinonasal epithelium samples from this study (haematoxalin and eosin [H&E] stain). Key structures are labelled: (Gl) glands, (B) basal cells, (V) vasculature, (Go) goblet cells and (C) ciliated columnar cells.

2. 3. 1 Sinonasal secretions and airway surface liquid (ASL)

The two main sources of sinonasal secretions in surface epithelium are submucosal glands and goblet cells (Figure 2.2). A small proportion of fluid also comes from the condensation of exhaled air from the lower airway, and from the drainage of tears via the nasolacrimal ducts.[168] Sinonasal glands are made up of serous and mucinous sub-glands lined with distinct cell types that drain via simple cuboidal ducts into common ducts. Serous glands outnumber mucinous by approximately 8:1 in sinonasal tissue. Mucinous and serous exudates mix and are secreted onto the airway lumen as airway surface liquid (ASL).

Figure 2.12: Schematic diagram of sinonasal secretions

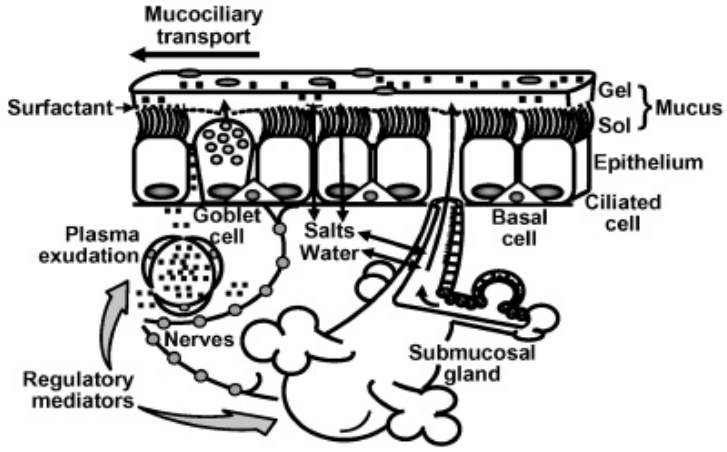

Figure 2.2: This diagram demonstrates the stimulation of submucosal gland and goblet cell secretion in sinonasal mucosa. Neural input stimulates both gland secretion and plasma exudation. Salt and water move across the surface and glandular epithelia, and mucus is secreted from the glands and exocytosed from the goblet cells. The image is reproduced with permission from Rogers[169]

Airway surface liquid (ASL) is a vital first line of mucosal defence in the airway.[166] The epithelium of the entire respiratory tract is coated in a dual-layer ASL. The top gel layer is a thick mucus blanket which acts as a physical protective barrier, while the lower one is a thin and watery sol layer or periciliary liquid (PCL).[166, 169] Periciliary liquid (PCL) allows the cilia to beat and carry the outer mucus layer away along the mucociliary clearance pathways.

Periciliary liquid (PCL) also contains innate immune antimicrobial peptides. While cilia are present to move the heavier, top layer of ASL, they will not function if inadequately lubricated by the PCL at their base.[155]

One to two litres of ASL is produced daily, consisting of glycoproteins (2–3%), salts (2–3%) and water (95%).[161] Mucins are heavily glycosylated proteins encoded by MUC genes and are a major ASL protein constituent.[169] Other proteins in ASL include: lactoferin, lysozyme, antitrypsin, transferrin, lipids and cytokines.[153]

Mucins are synthesised intracellularly and stored in mucin granules in a condensed form, typically occupying more than 75% of the cell's cytoplasm.[166] When mucin secretion is stimulated by any one of the neural mediators known to signal goblet cells, the granular vesicle is exocytosed and releases mucin into the gland lumen (Figure 2.3).[170, 171] When exposed to water, the mucin proteins then undergo a rapid

transformational change, exchanging bonded sodium ions for calcium. Mucin then bonds to many water molecules, unfolding and increasing up to 600 times its original size. The viscoelastic properties of ASL (i.e. how thick or thin it is) are dependent on how much water is available to saturate secreted mucin proteins.

Figure 2.13: Mucin exocytosis and protein conformation change

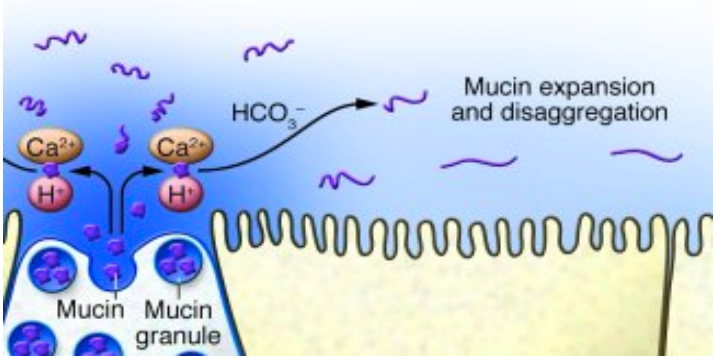

Figure 2.3: This diagram demonstrates the unfolding and massive expansion of mucin proteins after exocytosis and exposure to water. The protein donates sodium ions in favour of calcium ions and its affinity to bond with water increases rapidly. The image is reproduced with permission from De Lisle[172]

Extensive studies of sodium and chloride ion concentration in ASL have determined that the osmotic gradient produced by the salt load at the epithelial lumen drives water transport.[173, 174] Sodium is secreted into the gland lumen via the cystic fibrosis transmembrane conductance regulator (CFTR) cell membrane protein, following the Cl-gradient and creating a hyperosmolar stimulus for water to be transported into the gland lumen.[175, 176]

Water is harvested from the submucosal vascular plexus for ASL.[177, 178] The glandular epithelial cells control hydration via transepithelial water transport at the gland luminal surface in response to the glandular luminal osmotic gradient.[179] AQP5 is a rate-limiting step in the regulation of water secretion into the airway gland.[120] In addition, localised hypertonic stimulus increased AQP5 expression in the epithelial lumen in experimental conditions, increasing luminal water transport via AQP5 in response to a high osmotic gradient.[180]

2. 3. 2 Regulation of ASL

Both the volume and composition of ASL are dependent on water, ions and proteins transported across the epithelium, and on the rate at which the water evaporates from the ASL.[170] Water is constantly transported into and out of the ASL via the surface epithelium.[152, 181] No difference in ASL composition between the nose and paranasal sinuses has been observed.[182-184]

The mucus layer appears to act as the fluid reservoir, and its volume fluctuates within a broad homeostatic range, donating or absorbing fluid to maintain a constant PCL volume.[183] Regulation of ASL hydration is partially understood and two regulatory hypotheses have been proposed:[184] The "compositional" hypothesis states that active ion transport regulates ASL volume.[183] Boucher et al. found that normal ASL sodium concentration is <50 mM (i.e. less than sodium plasma concentration), creating an osmotic gradient across the epithelium which requires active regulation.[182] This hypotonic ASL state has also been shown to be the optimal range for the defensin proteins that provide innate immunity in the ASL.[185] In measuring the PCL and mucus layer height and tracking active epithelial ion transport, Boucher et al. observed apparent epithelial self-regulation of PCL height.[183] It was also found that active transport of sodium into the airway lumen increases overall ASL volume.[184] When ASL height approached the same height as the cilia, active transport switched to increased chloride secretion, resulting in slowed sodium and water absorption from the lumen.[182] This process is highly sensitive to experimental sodium channel blockade.[183] An amiloride blockade of sodium reabsorption increased the total ASL height.[174] The mucus layer volume increased, sequestering excess water as a reservoir, while the PCL height remained constant.[184]

The "volume" hypothesis attributes continuous airway secretion to hydrostatic force.[186] The hypothesis states that hydrostatic force on the sub-epithelium is responsible for pushing water into the airway across the epithelium, and that other factors such as Donnan forces, surface tension and/or ciliary recognition of ASL height all facilitate regulation of total ASL volume. The knowledge of the factors contributing to the "volume' hypothesis is limited because many are measured indirectly.

2. 4 Transepithelial water permeability and transport

Tissue water permeability (P_f) is defined as the volume of water that moves through a membrane per unit time, per unit area and per unit of transmembrane pressure.[187] The movement of fluid between body compartments and across mucosal surfaces is integral to mammalian homeostasis, with the human body moving 200 litres across cell membranes in a day[188]. However, P_f regulation is a complex process that remains incompletely understood.[189]

The three types of epithelial P_f are : high constitutive osmotic or "leaky" (e.g. proximal renal tubules, choroid plexus and small intestine); low constitutive (e.g. urinary tract); and variable or "regulated" (e.g. vasopressin-sensitive renal collecting duct).[190] Electrolyte transport has been extensively studied, and knowledge of water molecule movement through human tissue is progressing.[179, 188, 189, 191, 192]

Basic principles dictate that water moves across the epithelium by both passive and active mechanisms.[190, 193] Several mechanisms have been identified in transepithelial water transfer [194] with current theory including four transport mechanisms: passive transcellular (diffusion); passive paracellular; active transcellular (including AQP-mediated transport); and combination (transcellular then paracellular) (Figure 2.4).[191] The study of this area is limited by the inherent difficulty in measuring the flow of water by separate mechanisms.

Figure 2.14: Proposed pathways of transepithelial water transport

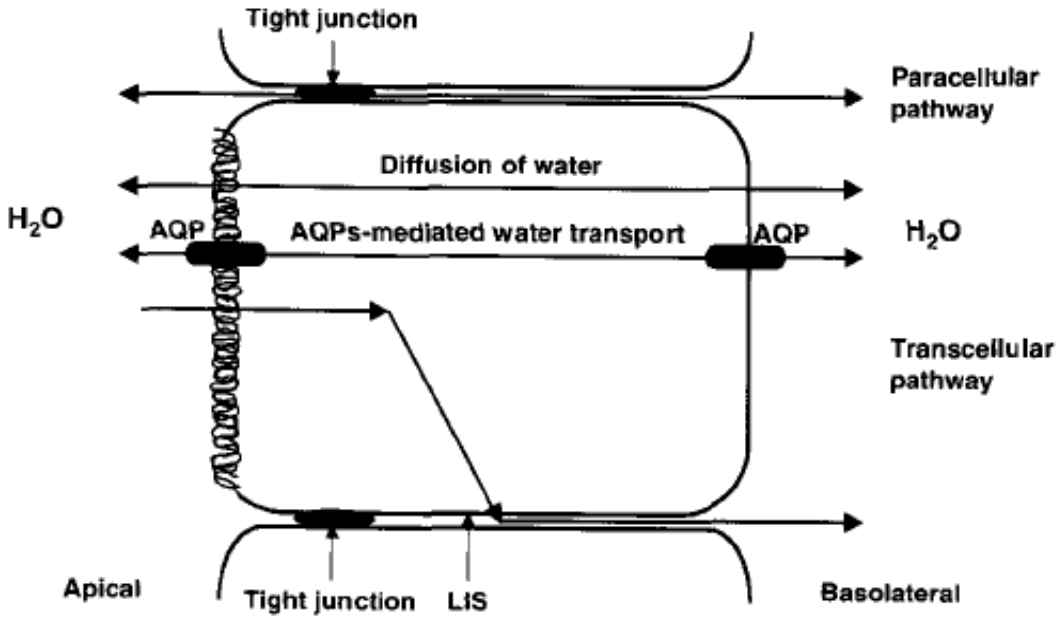

Figure 2.4: This diagram incorporates the four transepithelial water transport mechanisms: passive transcellular (diffusion); passive paracellular; active transcellular (including AQP-mediated transport); and combination. The image is reproduced with permission from Masyuk et al.[192]

2. 4. 1 Passive, transcellular water movement: Diffusion

Passive movement (or diffusion) is the extensively studied phenomenon of small, uncharged molecules moving across a membrane at a low but measurable rate.[195] The conventional understanding is that Fick's law determines water molecule movement through tissue: molecules pass from an area of high water molecule concentration, through a semi-permeable membrane, to an area of lower concentration, following a negative concentration gradient.[193]

At the cellular membrane level, diffusion is rate-limited by the hydrophobic hydrocarbon region of the cell membrane lipid bi-layer.[196] While it is not immediately obvious that water would be able to interact with hydrophobic lipid and cross a bi-layer membrane, Finkelstein's solubility-diffusion model describes a proportional relationship between the membrane lipid content, the membrane's lack of fluidity and its water permeability. Therefore, the higher the membrane lipid content, the slower the diffusion rate. This hypothesis has been confirmed by subsequent studies.[197] Investigation continues into the limitations of the solubility-diffusion model, particularly as the understanding of active transport pathways improves.[198]

2. 4. 2 Passive, paracellular water movement

Experiments have successfully reproduced transcellular water transport; however, paracellular water transport remains more controversial.[190, 192, 199] Indirect observations of paracellular water transport have proposed that water has the capacity to move from the interstitum between adjacent cells, transit the intercellular space via tight junctions, and reach the epithelial lumen surface.[200]

Tight junctions form a belt-like network of intercellular connections, projecting from one epithelial cell membrane and binding to the adjacent cell.[167] These junctions restrict paracellular movement and form the fluid compartment boundaries between tissues. It is believed that tight junctions are somewhat water permeable but quantifying this form of water movement is challenging.[191] Paracellular ion, solute and large, polar molecule movements have been quantified, and it is hypothesised that solvent drag promotes water to follow these molecules; however, there has been little direct study of water molecules.[192]

2. 4. 3 Active transcellular water transport

Discovery of AQPs have revolutionised the understanding of active transcellular water transport[3]; however, other active mechanisms are also involved in transcellular water transport (as discussed below).[189, 190] Epithelial water permeability is heavily influenced by the cell membrane components that participate in active water molecule transport and, as mentioned, this varies anatomically and based on tissue function. The AQP water transport described above is responsible for part of daily water movement; however, more recent studies have demonstrated that other co-transporters and uni-porters make a significant contribution while simultaneously transporting other molecules.[179, 188] Many ion transporters, such as glucose transporters and glucose/sodium co-transporters, move water across cell membranes as an intrinsic part of their molecular transport mechanisms. Other groups of researchers consider that the movement of water with molecular transport is the result of osmosis.[201] The experimental challenges of measuring the movement of small volumes of water molecules across the cell membrane means that, while each hypothesis can be tested indirectly, it has not yet been possible to separate out water movement due to osmosis across the cell membrane from active transport via ion transporters; thus, debate continues in this area.[190]

2. 4. 4 Sinonasal water transport

There has been no published work specific to the water permeability of normal sinonasal tissue. Pedersen et al. investigated the water permeability of an upper airway epithelial explant model harvested from nasal polyps.[187] They showed that water movement is in the same range as that known for mammalian AQPs. Unfortunately, their model was based on diseased nasal polyp tissue and no normal sinonasal tissue was used for comparison. The same group of researchers went on to demonstrate that water permeability of the nasal polyp tissue increased at a rate paralleled by AQP5 expression after exposure to hyperosmotic conditions.[202] They showed that any activation or blockade of glucose or combined sodium/glucose transporters did not yield any change to water movement, suggesting these other transporters play little role in the overall movement of water in the sinonasal epithelium.

Chapter 3. AQPs in the respiratory tract

3. 1 AQPs in the respiratory tract

There is ongoing debate regarding fluid movement through respiratory tissue.[203] Initial studies suggested that respiratory tract AQPs were vital to all water transport; however, subsequent studies were not able to demonstrate significant changes to respiratory tissue hydration or water movement in AQP-null mice, with the exception of secretory glands.[120, 204-206] Studies of respiratory tract distribution of some mammalian AQPs have been undertaken in humans, rats and mice.[5, 207] Eight key studies are summarised in Table 3.1, highlighting that only some of the 13 known AQPs, namely, AQP1, AQP3, AQP4, AQP5 and AQP8 appear to be localised to the respiratory tract.[60, 67, 79, 81-83, 208, 209]

Table 3.1: Expression and localisation of AQPs in respiratory tract

Tissue Type	Human	Human	Rat	Rat	Rat	Rat	Mouse	Mouse
Technique	*In situ hybridization, IF*	*IHC, histomorphometric analysis*	*IHC, IEM*	*Electrophoresis, immunoblots*	*RT-PCR, IHC, IEM*	*RT-PCR, IHC*	*IHC, IF, IP, IEM*	*RT-PCR, IHC*
AQP1	**Kreda et al**	**Mobasheri et al**	**Nielsen et al**	**King et al**	**Funaki et al**	**Elkjaer et al**	**Matsuzaki et al**	**Song et al**
Endothelium	N/A	+ (Lung, bronchus)	+ (Bronchus, trachea nasopharynx)		N/A	N/A	-	+ (Visceral and parietal pleura)
Fibroblast/connective tissue	N/A	-	+		N/A	N/A	-	-
Inflammatory cells	N/A	-	-		N/A	N/A	-	-
				No cellular localisation in study, only region				
Surface epithelium								
Basolateral	N/A	-	-		N/A	N/A	-	-
Apical	N/A	-	-		N/A	N/A	-	-
Glandular epithelium								
Basolateral	N/A	-	-		N/A	N/A	-	-
Apical	N/A	-	-		N/A	N/A	-	-
Other				+ (Nasopharynx, trachea, bronchi, bronchioles)			+ (Mesothelial cells of visceral pleura)	+ (Mesothelial cells of visceral pleura)
AQP2	**Kreda et al**	**Mobasheri et al**	**Nielsen et al**	**King et al**	**Funaki et al**	**Elkjaer et al**	**Matsuzaki et al**	**Song et al**
Endothelium	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Fibroblast/connective tissue	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Inflammatory cells	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Surface epithelium								
Basolateral	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Apical	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Glandular epithelium								
Basolateral	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Apical	N/A	N/A	N/A	N/A	N/A	N/A	-	N/A
Other								
AQP3	**Kreda et al**	**Mobasheri et al**	**Nielsen et al**	**King et al**	**Funaki et al**	**Elkjaer et al**	**Matsuzaki et al**	**Song et al**
Endothelium	-	N/A	-		N/A	N/A	-	N/A
Fibroblast/connective tissue	-	N/A	-		N/A	N/A	-	N/A
Inflammatory cells	-	N/A	-		N/A	N/A	-	N/A
Surface epithelium								
Basolateral	+ (Bronchi, bronchioles, nose)	N/A	+ (Trachea, nose, nasopharynx)		N/A	N/A	+ (Trachea, bronchioles)	N/A
Apical	+ (Bronchioles)	N/A	-		N/A	N/A	-	N/A
Glandular epithelium								
Basolateral	+ (Bronchi, bronchioles, nose)	N/A	-		N/A	N/A	-	N/A
Apical	-	N/A	-		N/A	N/A	-	N/A
Other	+ (Basolateral Type II			+ (Nose, trachea)				

Tissue Type	Human	Human	Rat	Rat	Rat	Rat	Mouse	Mouse
Technique	*In situ hybridization, IF*	*IHC, histomorphometric analysis*	*IHC, IEM*	*Electrophoresis, immunoblots*	*RT-PCR, IHC, IEM*	*RT-PCR, IHC*	*IHC, IF, IP, IEM*	*RT-PCR, IHC*
AQP4	**Kreda et al**	**Mobasheri et al**	**Nielsen et al**	**King et al**	**Funaki et al**	**Elkjaer et al**	**Matsuzaki et al**	**Song et al**
Endothelium	-	N/A	-		N/A	N/A	-	N/A
Fibroblast/connective tissue	-	N/A	-		N/A	N/A	-	N/A
Inflammatory cells	-	N/A	-		N/A	N/A	-	N/A
Surface epithelium								
Basolateral	-	N/A	+ (Bronchi, trachea, nasopharynx)		N/A	N/A	++ (Bronchioles), + (Trachea)	N/A
Apical	-	N/A	-		N/A	N/A	-	N/A
Glandular epithelium								
Basolateral	+ (Bronchi)	N/A	+ (Nose)		N/A	N/A	-	N/A
Apical	-	N/A	-		N/A	N/A	-	N/A
Other				+ (Nasopharynx, trachea, bronchi, bronchioles)				
AQP5	**Kreda et al**	**Mobasheri et al**	**Nielsen et al**	**King et al**	**Funaki et al**	**Elkjaer et al**	**Matsuzaki et al**	**Song et al**
Endothelium	-	N/A	-		-	N/A	-	N/A
Fibroblast/connective tissue	-	N/A	-		-	N/A	-	N/A
Inflammatory cells	-	N/A	-		-	N/A	-	N/A
Surface epithelium								
Basolateral	-	N/A	-		-	N/A	+ (Bronchioles, Trachea)	N/A
Apical	+ (Nose)	N/A	-		-	N/A	+ (Trachea)	N/A
Glandular epithelium								
Basolateral	-	N/A	-		-	N/A	-	N/A
Apical	+ (Nose, Bronchus)	N/A	+ (Upper airway, salivary glands)		-	N/A	-	N/A
Other	+ (Type I pneumocytes)		+ (Apical Type I pneumocytes)	+ (Nasopharynx, trachea, bronchi, bronchioles, salivary gland)	+ (Apical Type I pneumocytes)		+ (Apical Type I and II pneumocytes)	
AQP8	**Kreda et al**	**Mobasheri et al**	**Nielsen et al**	**King et al**	**Funaki et al**	**Elkjaer et al**	**Matsuzaki et al**	**Song et al**
Endothelium	N/A	N/A	N/A	N/A	N/A	-	N/A	N/A
Fibroblast/connective tissue	N/A	N/A	N/A	N/A	N/A	-	N/A	N/A
Inflammatory cells	N/A	N/A	N/A	N/A	N/A	-	N/A	N/A
Surface epithelium								
Basolateral	N/A	N/A	N/A	N/A	N/A	+ (Trachea, bronchus)	N/A	N/A
Apical	N/A	N/A	N/A	N/A	N/A	-	N/A	N/A
Glandular epithelium								
Basolateral	N/A	N/A	N/A	N/A	N/A	+ (Myoepithelial cells of Trachea, bronchi)	N/A	N/A
Apical	N/A	N/A	N/A	N/A	N/A	-	N/A	N/A
Other								

Table 3.1: The table includes eight key studies of AQPs in the respiratory tract: two in human tissue[82, 208]*; four studies of rat tissue*[60, 67, 79, 81]*; and two mouse studies* [83, 204] *The techniques used in the studies comprised: in situ hybridisation; immunofluorescence (IF); immunohistochemistry (IHC); histomorphometric analysis; immunoelectron microscopy (IEM); electrophoresis; immunoblots; RT-PCR; and immunoperoxidase labelling. Scores: - = not localised, +=localised at site. N/A indicates no data regarding AQP expression are available at that site.*

3. 1. 1 AQPs in rat and mouse respiratory tracts

AQP1–AQP5 and AQP8 have been localised in the respiratory tracts of rats and mice using a variety of experimental techniques (Table 3.1, Figure 3.1). AQP1 has been localised to the respiratory microvascular endothelium, consistent with findings in which it has been localised elsewhere in the body.[67, 81, 83, 204] To date, no studies have identified the expression of AQP2 protein in the respiratory tract.[5, 83] AQP3 was identified in the basement membrane of the nose, tracheal and bronchial surface epithelium, and AQP4 was seen with the same distribution and additional expression in the smaller airways (down to bronchioles).[67, 81, 83] AQP5 has been localised in the nose, trachea, bronchus, bronchioles and salivary glands.[81] Other studies found a strong expression of AQP5 in the distal airways and within the alveoli.[67, 79, 204] AQP8 was identified as localised to the basement membrane of the tracheal and bronchial surface epithelium, and in myoepithelial cells surrounding glands in the same areas.[60]

Figure 3.1: AQP expression in the respiratory tract

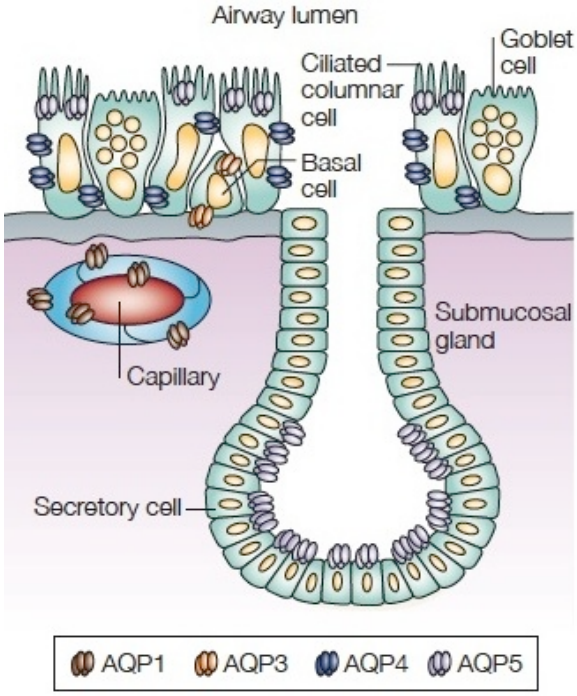

Figure 3.1: This diagram demonstrates the expression and localisation of AQPs in the rat respiratory tract. AQP1 is seen in the endothelium of the local microvasculature. AQP4 transports water from the interstitum into the epithelial cells (both columnar and glandular cells) and water exits into the lumen via AQP5 on the cellular apex. The image is reproduced with permission from King et al.[10]

3. 1. 2 AQPs in the human respiratory tract

AQP expression in human respiratory tissue has been the subject of only a few studies.[82, 208, 210] Most knowledge of respiratory tissue AQP localisation and function has been extrapolated from mouse and rat tissue studies (Figure 3.1).

Kreda et al. identified AQP3, AQP4 and AQP5 in nasal, bronchus, bronchiolar and alveolar tissue in correlation with previous studies of rat and mice tissue.[82] AQP3 was expressed along the length of the respiratory tract: the basolateral membranes of surface and glandular epithelia of the nose and bronchi, and apical membranes of the same cells in proximal and terminal bronchioles while, in alveolar tissue, AQP3 was identified in type II pneumocytes. AQP4 was only localised at the bronchial basolateral membrane of glandular epithelium and in type I pneumocytes. AQP5 was found at the apical surface of nose surface epithelium, Type I pneumocytes and the apical surface of nose and bronchus glandular epithelium.

Mobasheri et al. investigated AQP1 expression throughout the body, but only localised AQP1 at the vascular endothelium of the respiratory tract.[208] King et al. found that permeability of this vascular tissue was decreased in AQP1-null humans.[210] Only a single study of AQP-mediated tissue water permeability of human respiratory tissue was identified in the literature: the Pedersen study of AQP function in tissue harvested from nasal polyps (discussed in Section 2.4.4).[202]

3. 1. 3 Hypothesised role of AQPs in respiratory tract homeostasis

No published body of work has summarised the role and function of AQPs in the respiratory tract. The following section presents published literature that has investigated several aspects of respiratory tract homeostasis involving AQPs.

A small number of studies investigated the role of AQPs in fluid movement across respiratory and lung tissue using a range of experimental animal models.[120, 204, 206] Studies of the contribution that some AQPs make to overall fluid homeostasis in respiratory tissue found that only limited interruption to normal function was seen when AQPs were knocked out (i.e. mice selectively bred without expression of a particular AQP).[120, 204-206] Unexpectedly, airway humidification, airway surface liquid (ASL) hydration and fluid absorption were largely unchanged in the absence of AQP5[204-206]; however, glandular secretions were significantly different.[120] Gene knockout studies in mice identified AQP5 as the rate-limiting factor in sinonasal submucosal gland secretion (nasal septum, nasal cavity, maxillary sinus and posterior nasopharynx).[120] Apical AQP5 glandular epithelial cells were

identified, with basolateral AQP4 expression in the same cells. AQP5-null mice produced hyper-concentrated and viscous submucosal gland secretions compared to normal and AQP4-null mice; however, glandular structure and density were unchanged. The secretions contained less than 50% of normal fluid volume, indicating significantly reduced water transport.

Song et al. labelled AQP1, 3, 4 and 5 in the respiratory tract of mice in a series of experiments, using a number of novel techniques to assess water movement across airway tissue.[204] A 5% decrease in upper and lower airway humidification was observed in AQP3/AQP4 dual-null mice and AQP1/AQP5 dual-null mice. ASL thickness and ASL [Na+] were unchanged in AQP3/AQP4-null mice tracheas compared to normal (not studied in the AQP1/AQP5 mice). AQP3-null mice had a 35% reduction in upper airway tissue water but their net epithelial water absorption remained unchanged (no change was found for AQP4).

The Verkman group published a series of lung fluid transport studies on AQP1-, AQP4- and AQP5-null mice.[205, 206] No changes in lung tissue morphology in the AQP-null mice were identified; however, lung tissue water permeability (P_f) was reduced by 10-fold in AQP1 and AQP5 (no AQP4 was found to be expressed in the distal lung epithelium in the experiments). Despite this, no overall change was observed in omostically driven water transport for either AQP, indicating that tissue P_f was not the regulating factor for the rate of respiratory tissue fluid transport. No resultant tissue oedema was observed following decreased AQP water transport.

Several regulatory factors have been associated with AQP5 in respiratory tissue: TNF-alpha was found to inhibit AQP5 in mouse tissue[211]; cyclic adenosine monophosphate (cAMP) increased AQP5 during and after transcription (the protein kinase A pathway was implicated)[86]; and AQP5 induction was increased in the presence of hypertonicity. [180] AQP3 was found to be inhibited in the presence of nickel and low pH[43] while the CFTR positively regulated AQP3 in the airway epithelium.[212] Research continues in this area.

Overall, AQPs have been implicated in respiratory tract secretion hydration but more work is required to establish the additional contributions of AQPs in respiratory tissue homeostasis.

3. 2 AQPs in normal sinonasal tissue

3. 2. 1 AQPs in human sinonasal tissue

Four published studies have reported AQP localisation in human sinonasal tissue, as summarised in Table 3.2.[82, 213-215] Following the discovery of more AQPs, the establishment of normal AQP expression in human sinonasal tissue will be a valuable step in understanding normal sinonasal tissue homeostasis.

Table 3.2: Expression and localisation of AQPs in sinonasal tissue

Tissue Type	Human	Human	Human	Human	Rat	Rat
AQP1	**Seno et al**	**Kreda et al**	**Shikani et al.**	**Altuntas et al**	**Ablimit et al**	**Nielsen et al**
Endothelium	+	N/A	N/A	-	+	+
Fibroblast/connective tissue	+	N/A	N/A	+/-	+	+
Inflammatory cells	N/A	N/A	N/A	N/A		
Polyps	N/A	N/A	N/A	++	N/A	N/A
Surface epithelium						
Basolateral	-	N/A	N/A	-	-	-
Apical	-	N/A	N/A	-	-	-
Glandular epithelium	-	N/A	N/A	-	-	-
AQP2	**Seno et al**	**Kreda et al**	**Shikani et al.**	**Altuntas et al**	**Ablimit et al**	**Nielsen et al**
Endothelium	-	N/A	N/A	N/A	N/A	N/A
Fibroblast/connective tissue	-	N/A	N/A	N/A	N/A	N/A
Inflammatory cells	N/A	N/A	N/A	N/A	N/A	N/A
Polyps	N/A	N/A	N/A	N/A	N/A	N/A
Surface epithelium	+ (cytoplasm)					
Basolateral	-	N/A	N/A	N/A	N/A	N/A
Apical	-	N/A	N/A	N/A	N/A	N/A
Glandular epithelium	+	N/A	N/A	N/A	N/A	N/A
AQP3	**Seno et al**	**Kreda et al**	**Shikani et al.**	**Altuntas et al**	**Ablimit et al**	**Nielsen et al**
Endothelium	-	-	N/A	N/A	-	-
Fibroblast/connective tissue	-	-	N/A	N/A	-	-
Inflammatory cells	N/A	N/A	N/A	N/A	N/A	N/A
Polyps	N/A	N/A	N/A	N/A	N/A	N/A
Surface epithelium					+ (olfactory)	
Basolateral	+	++ (Basolateral)	N/A	N/A	+	+
Apical	-	-	N/A	N/A	+/-	-
Glandular epithelium	+ (Basolateral)	+ (Basolateral)	N/A	N/A	+ (Basolateral)	+ (Basolateral)
AQP4	**Seno et al**	**Kreda et al**	**Shikani et al.**	**Altuntas et al**	**Ablimit et al**	**Nielsen et al**
Endothelium	-	-	N/A	-	-	-
Fibroblast/connective tissue	-	-	N/A	-	-	-
Inflammatory cells	N/A	N/A	N/A	N/A	N/A	N/A
Polyps	N/A	N/A	N/A	-	N/A	N/A
Surface epithelium						
Basolateral	-	-	N/A	-	++	-
Apical	-	-	N/A	-	++	+
Glandular epithelium	+ (Basolateral)	-	N/A	-	-	+ (Basolateral)
AQP5	**Seno et al**	**Kreda et al**	**Shikani et al.**	**Altuntas et al**	**Ablimit et al**	**Nielsen et al**
Endothelium	-	-	N/A	N/A	-	-
Fibroblast/connective tissue	-	-	+/-	N/A	-	-
Inflammatory cells	N/A	N/A	N/A	N/A	N/A	N/A
Polyps	N/A	N/A	+	N/A	N/A	N/A
Surface epithelium						
Basolateral	-	-	+	N/A	-	-
Apical	-	++	+	N/A	-	-
Glandular epithelium	+ (Apical)	++ (Apical)	+ (Apical)	N/A	+	+ (Apical)

Table 3.2: The table presents a summary of known AQP expression in normal sinonasal mucosa collated from the published literature. Scores: - = not localised, + =localised, ++ =strongly expressed at this site. N/A indicates no data regarding AQP expression are available at that site.

Seno et al. investigated AQP1–AQP5 expression in human sinonasal tissue (inferior turbinate) using semi-quantitative RT-PCR and immunohistochemistry in six normal controls[213]: (1) AQP1 in vasculature endothelium and sub-epithelial fibroblasts; (2) AQP2 in the cytoplasm of surface epithelial cells; (3) AQP3 at the basolateral membrane of surface and glandular epithelial cells; (4) AQP4 at the basolateral membrane of glandular epithelial cells; and (5) AQP5 at the apical membrane of glandular epithelial cells. This study also looked at (6) AQP expression differences in allergic rhinitis and CRS (see Section 5.1.2). Overall, prior to our study, this study was the most comprehensive study of AQP expression in human sinonasal tissue. Localisation of AQP2 in sinonasal tissue was a novel finding (no other study in any species has reproduced this finding); however, other AQP localisation was similar to the known respiratory tract AQP expression.

Kreda et al. examined AQP3, AQP4 and AQP5 throughout the human respiratory tract, including sinonasal tissue harvested from the turbinate using *in situ* hybridisation and immunofluorescence.[82] AQP3 was localised to the basolateral membrane of surface and glandular epithelial cells, AQP4 was not detected, and AQP5was localised at the apical membrane of surface and glandular epithelial cells. Their study also investigated AQP expression profiles at other sites in the respiratory tract, with results consistent between sinonasal tissue and other airway locations. Their results reflected the distinct structure of alveolar epithelium when compared to the rest of the respiratory tract.

Shikani et al. examined AQP5 expression and localisation in sinonasal tissue using semi-quantitative RT-PCR and immunohistochemistry in five normal controls.[214] AQP5 was localised to surface and glandular epithelium, consistent with the published literature. Altuntas et al. investigated AQP1 and AQP4 expression in normal human turbinate tissue by immunohistochemistry in 10 patients.[215] AQP1 was identified in connective tissue fibroblasts, and no AQP4 was seen at any site. Finally, a fifth study, Jun et al. used semi-quantitative PCR and cell culture to confirm AQP3 and AQP4 expression in human turbinate tissue, but no AQP protein was localised to specific anatomical sites.[216]

3. 2. 2 AQPs in rat sinonasal tissue

Three published rat studies have also reported localisation of AQPs to sinonasal tissue.[67, 81, 217]

Ablimit et al. used immunohistochemistry to identify AQP0–AQP10 in normal rat nasal mucosa.[217] They successfully identified AQP1 in vasculature endothelium and in connective tissue. AQP3 was found in olfactory epithelium, the basal cells of surface epithelium and the basolateral surface of glandular epithelium. AQP4 was strongly localised to the olfactory surface epithelium and basolateral membranes of glandular epithelium. Finally, AQP5 was localised to the apical membranes of glandular epithelium. No other AQP protein was successfully localised.

Neilsen et al. also studied AQP1, AQP3, AQP4 and AQP5 in rat nasal tissue using immunohistochemistry and immunoelectron microscopy.[67] Low-grade expression of AQP1 was seen in connective tissue and vascular endothelium; basolateral expression of AQP3 was in both surface and glandular epithelial cell membranes; AQP4 was at the apex of surface epithelial cells and basolateral surfaces of glandular cells; and AQP5 was localised only in the apical membrane of glandular epithelium.

King et al. investigated AQP1, AQP3, AQP4 and AQP5 in the respiratory tract of rats using electrophoresis and immunoblotting, successfully labelling each AQP.[81] This study did not localise AQP proteins; therefore, it was not included in the summary table.

The studies of AQP expression in rat tissue correlated very closely, with only one contrast: AQP4 was present in glandular epithelium in one study, but not in the other. When compared to human AQP tissue localisation, there is close correlation of the findings.

AQP1:	Connective tissue (all studies correlate)
	Vasculature (only one study unable to localise AQP1 here)
AQP3:	Surface and glandular epithelium (all studies correlate)
AQP4:	Surface epithelium (only localised here in rats)
	Glandular epithelium (only localised here in two studies: human and rat)
AQP5:	Surface epithelium (only localised here in two human studies)
	Glandular epithelium (all studies correlate)

There are limitations when extrapolating protein expression studies from rodent models to humans, [203, 218, 219] Key AQP scientists have cautioned against the overemphasis of animal model findings. They acknowledge important differences in

AQP expression patterns between humans and rodents, and have strongly advocated more work in human tissue, searching for new AQPs and further investigation of AQP function in respiratory tissue.[203, 218, 219] Despite this, only minor differences in AQP4 and AQP5 localisation were seen between the groups in this review of the literature. Overall, the published literature indicates that AQP1, AQP2, AQP3, AQP4 and AQP5 are likely to be expressed and localised in normal human sinonasal tissue, while no definitive study has been done to investigate other AQPs.

Chapter 4. Chronic rhinosinusitis

4. 1 Chronic rhinosinusitis

4. 1. 1 Definition

Chronic rhinosinusitis (CRS) is a chronic inflammatory condition with a diagnosis defined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 (EPOS 2012) based on clinical symptoms and investigation findings.[220] Major diagnostic criteria for CRS are: nasal congestion/obstruction and/or anterior or posterior nasal discharge. Minor criteria are: facial pain or pressure and reduction or loss of sense of smell. Examination findings include: endoscopic evidence of polyps, mucopurulent discharge, mucosal oedema or obstruction of the middle meatus. Phenotypically, CRS is classified into disease with nasal polyps (CRSwNP) and disease without nasal polyps (CRSsNP). There is increasing evidence of further complex heterogeneity between patient groups (e.g. neutrophilic vs. eosinophilic polyps).[221-224]

4. 1. 2 Epidemiology

Objective diagnosis of CRS is difficult without specialised equipment; therefore, it is difficult to ascertain accurate epidemiological data.[220, 225-229] Correlation between objectively diagnosed CRS and self-report varies significantly.[225] Depending on the inclusion criteria, CRS is estimated to affect 2–15% of the population in their lifetime, and rates of CRS vary geographically and by ethnicity.[230] CRS affects up to 10% of the Australian population at any time.[231] It also impacts on quality of life[232, 233]: those with CRSwNP report a greater burden of disease.[234] Achieving symptom control in CRS is difficult, and treatments (medical and surgical) can be expensive.[235] The cost to the community is significant in terms of health care expenditure, lost productivity and decreased workforce participation.[236-238]

4. 1. 3 Aetiology

Chronic rhinosinusitis (CRS) aetiology remains unclear; however, it is hypothesised to be a heterogeneous group of inflammatory processes arising in sinonasal mucosa due to a defective host immune barrier response to environmental triggers.[220, 239-242] CRS is likely to represent a spectrum of disease, a collection of separate pathological processes, linked by common final symptomatology.[243]

In CRS, there are pathological changes at cellular, inflammatory and immunological levels.[162, 239-241, 243-250] Dysfunction occurs at multiple levels: the sinonasal

epithelium, mucociliary clearance, innate and acquired immunological defences.[240] Pathogens encountered by normal sinonasal mucosa are effectively targeted without an inflammatory response.[162, 251, 252] In CRS, the host response is altered and inflammation results when pathogens infiltrate the airway surface liquid (ASL) and enter a damaged epithelial surface.[245] Over time, the epithelium becomes primed and develops a chronic inflammatory response. It is hypothesised that CRS patients have reduced ASL immune peptide function.[162] Current research into sinonasal epithelium colonisation, pathogen load, biofilms, superantigens and microbiome in CRS aims to improve the understanding of the perpetuated inflammatory response of CRS.[248-250] Other diseases involving abnormal host immune barrier function have been identified, such as ulcerative colitis, Crohn's disease, asthma and psoriasis.[240] In the context of this study, establishing the role of AQPs in normal epithelial homeostasis may add to the understanding of the altered epithelial immune response in CRS.

4. 2 Pathological features of CRS

4. 2. 1 Oedema

Oedema and hyperaemia are hallmark features of all inflammation.[253] Increased blood flow and vasculature permeability are physiological responses to inflammatory chemokines such as histamine, prostaglandins and bradykinin, released by activated inflammatory cells[157]. However, after an acute inflammatory response, sinonasal mucosa normally returns to its baseline function, whereas mucosal oedema continues abnormally in CRS.[221, 241] Sinonasal vasculature is highly responsive to local reflex and systemic neural control, and noxious irritants trigger parasympathetic and axonal reflex vascular engorgement. There is increased post-capillary vascular pressure, vasodilation and loosening of endothelial tight junctions, as well as increased exudation of plasma into the extravascular tissue, resulting in mucosal congestion and oedema.[157, 222] This is perpetuated by ongoing cytokine release.[152, 254] No published literature was identified confirming this hypothesis of hydrostatic force across a leaky vasculature (exuding more plasma in response to cytokines) in sinonasal mucosa in CRS. We predict that AQPs play a role.

Figure 4.1: Inflammatory oedema and mucus secretion in sinonasal mucosa

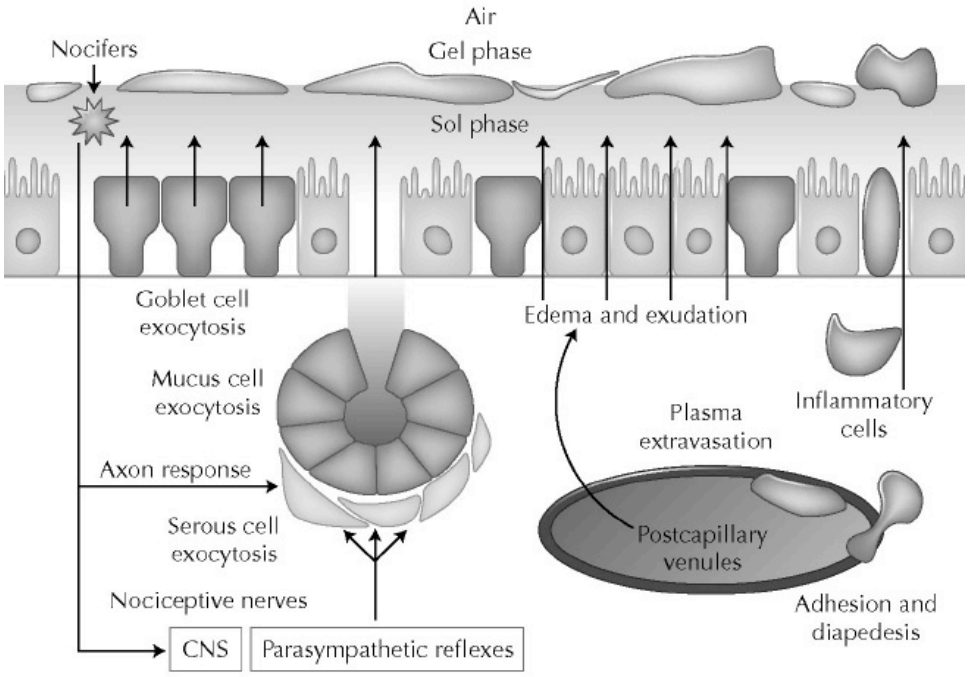

Figure 4.1: This diagram demonstrates: (a) parasympathetic regulation of mucus hypersecretion, and (b) neurally controlled response to an inflammatory agent and the resultant exudation of fluid from the vasculature, leading to oedema. The image is reproduced with permission from Yuta and Baraniuk[254]

4. 2. 2 Altered secretions

Thickened, viscous ASL is secreted in high volumes in CRS; however, the mechanism responsible for this is poorly understood.[254, 255] The drivers and regulation of ASL secretion are multifactorial, with a range of incompletely explored variables that determine the overall volume and viscosity of secretions. Mucus cell metaplasia is observed in other chronic airway diseases: this is a protective response by the epithelium to perpetual inflammation and harmful insults.[166] It involves conversion of airway ciliated and clara cells to goblet cells, resulting in more ASL secretion (possibly perpetuating the problem) in an attempt to dilute thick ASL containing inflammatory cytokines, dead/damaged cells and airway debris.[221, 256] The thicker, heavier ASL of CRS patients becomes stagnant within the nasal cavity and sinuses as a result of mucociliary dysfunction.[155, 163] Stasis is a recognised risk factor for pathogen proliferation and worsens and perpetuates mucosal inflammation due to prolonged mucosal exposure to pathogens.[256] Mucociliary dysfunction and

dyskinesia occur when sinonasal cilia fail to beat back and forth effectively to move ASL.[155] In CRS, normal mucociliary clearance is prevented, predisposing individuals to recurrent infection and chronic inflammation.[257] Epithelial oedema and thick, dehydrated ASL in CRS contribute to ciliary dysfunction as the cilia require specific PCL hydration conditions to function (as discussed in Section 2.3.1).[163, 258] Cystic fibrosis is similarly associated with ciliary dyskinesia due to the thick, viscous secretions that result from altered sodium transport in the glandular epithelial cells.[257] The lack of osmotic gradient across the glandular epithelium means that there is no driver for water transport into the gland lumen, resulting in sticky ASL. We hypothesise that a similar scenario arises in CRS, however, with a defect in the water transport proteins rather than the electrolyte transport. Cystic fibrosis is also associated with increased frequency of nasal polyps.[257, 259]
While hydration of ASL is key to its viscosity, no published literature was identified which directly addresses the pathway of water into ASL in sinonasal mucosa, its regulation or how this is altered in CRS. We hypothesise that AQPs play a key role in moving water across the sinonasal epithelium and maintaining ASL viscosity, and that there is an interruption of this process in CRS.

4. 2. 3 Tissue remodelling and polyp formation

The histological changes due to tissue remodelling that are seen in CRS sinonasal tissue are: basement membrane thickening, squamous epithelial metaplasia, deposition of collagen, lamina propria fibrosis, mucous gland hyperplasia, goblet cell hyperplasia and, for some patients, polyp formation (Figure 4.2).[247] There are some differences in the range of these features between CRSwNP and CRSsNP tissue.[223] CRSsNP tissue tends to be more fibrotic, while CRSwNP tissue is characterised by more oedema, albumin, plasma proteins and other cytokine accumulation and pseudocyst formation.[224] The collagen deposition patterns were also different, with excessive collagen produced and deposited in the extracellular matrix (ECM) of CRSsNP patients, while CRSwNP patients have a distinct lack of collagen associated with polyps, which is likely due to increased destruction.[224]

Figure 4.2: Histological changes in CRSsNP tissue

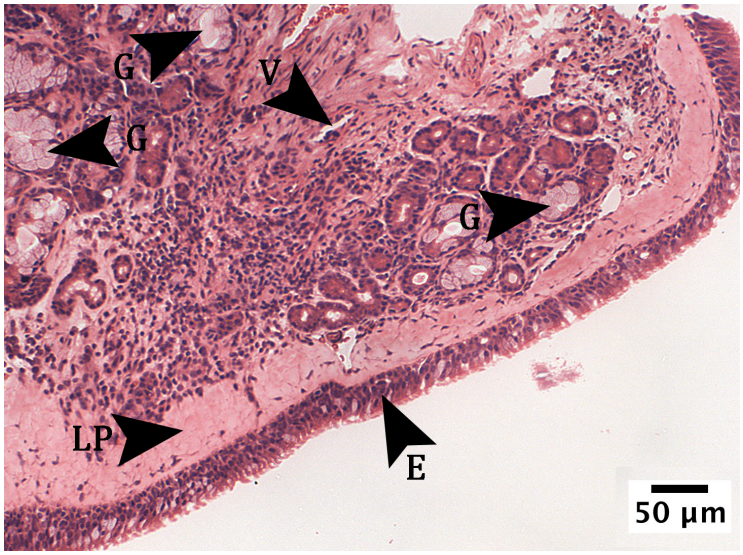

Figure 4.2: This photomicrograph is of one CRSsNP sinonasal tissue sample from this study (haematoxalin and eosin [H&E] stain). Key structures are identified: (E) epithelium; (G) mucus cell hyperplasia in glands; (V) vasculature; and (LP) lamina propria fibrosis.

Sinonasal polyps are gelatinous, oedematous out-pouching swellings of pedunculated nasal or sinus mucosa attached to the surrounding area by a stalk.[260] Compared to normal epithelium, histological features of a polyp are: atypical glandular formation, sub-epithelial oedema, fibrosis, goblet cell hyperplasia, mononuclear cell infiltration and variable grades of epithelial cell thinning and destruction.[222, 257, 261]

It is hypothesised that polyps result from chronic inflammation of the sinonasal mucosa.[221, 247] T_{H2}-mediated inflammation has been implicated in eosinophilic polyp formation in the Caucasian population[262] which differs from polyps in the Asian population which are more strongly neutrophilic, driven by T_{H1} and T_{H17}-mediated inflammation.[263, 264] In addition, polyps differed in Caucasian CRSwNP patients with asthma, who also had strongly eosinophilic inflammation.[265]

The study of polyp formation reveals two stages of tissue remodelling: early and mature.[266] An early polyp is made up of pseudocysts surrounded by looser connective tissue, fibroblasts and a leukocytes cap. Mast cells and myofibrocytes are adjacent to the polyp pedicle as it forms. In mature polyps, mast cells are diffusely distributed. Despite an extensive literature review, no studies were identified that had established the polyp water content or AQP expression in a non-cell cultured polyp.

Transport of water into and out of polyp tissue via either active or passive mechanisms remains uncharacterised.

4. 2. 4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) play a significant role in extracellular tissue remodelling in inflammation and have been studied in a range of diseases.[267] Matrix metalloproteinases (MMPs) are a group of calcium-activated, zinc-dependent proteolytic enzymes that target specific components of the extracellular matrix (ECM) and are responsible for reabsorption and repair of the ECM. Investigation of tissue remodelling and its regulation in CRS has identified an association with MMPs.[268, 269]

Matrix metalloproteinases (MMPs) are important in the maintenance of tissue integrity, playing key roles in normal cell proliferation, apoptosis, tissue remodelling and wound repair.[270] It is hypothesised that unregulated activity of MMPs contributes to localised tissue destruction, resulting in pseudocyst formation.[269] Matrix metalloproteinase (MMP) function is regulated by tissue inhibitors of metalloproteinases (TIMPs).[269] Classification of MMPs is based on the ECM target tissue of each enzyme.[271] Collagenases primarily degrade and regulate type I, II and III collagens in the ECM (including MMP-1, MMP-3, MMP-8 and MMP-13), while gelatinases digest the denatured collagen product, that is, gelatins (MMP-2 and MMP-9). Altered MMP and/or TIMP expression has been implicated in a wide range of pathological processes.[272]

In CRSwNP, altered expression of collagenase and gelatinase MMPs has been identified.[271] During early polyp formation, increased MMP-7 and MMP-9 were found in CRSwNP tissue.[269] In other CRSwNP studies, MMP-9 was the most commonly altered enzyme.[273-275] Bhandari et al. failed to identify a difference in MMP protein expression in CRSwNP; however, they localised MMP mRNA in polyp tissue of CRSwNP patients.[276] Finally, comparison of CRSsNP and CRSwNP tissue revealed differences in MMP-1, MMP-2 and MMP-8 expression between the groups, along with TIMP-2.[277]

Substantial study has been undertaken to target MMP function with pharmacological therapies, principally in cancer and other inflammatory disease.[278] The in-depth structural analysis of MMPs has identified possible molecules to target their function; however, the early work in cancer metastases has been disappointing due to the non-specificity of the medications and side-effect profiles.[279] Some promising

study of inflammation modulation by medications targeting MMPs has been done; however, a more complete understanding of altered MMP regulation in inflammation will facilitate better medication design.[280]

The current mainstays of CRS medical therapy are corticosteroids and antibiotics.[220] In CRSwNP patients treated with oral corticosteroid, MMP-2 and TMP-1 were reduced in polyp tissue and serum samples.[281] Matrix metalloproteinases (MMPs) are also the known target of tetracyclines.[282] Preliminary investigation in CRS has shown a reduction in MMPs in nasal secretions and polyp tissue after doxycycline treatment.[283] In CRSwNP patients treated with either oral steroid or doxycycline, a significant reduction in polyp size and symptoms was seen in both treatment groups; however, a significant decrease in MMP-9 expression was only seen in the group treated with doxycycline.[282] These findings are exciting and may lead to an improved understanding of the medications that better target the underlying pathophysiology in CRS.

Investigation into the association between AQPs and MMPs has been limited to date.[115, 132, 284, 285] Both have been associated with tissue remodelling as discussed earlier; however, a discrete mechanism linking these proteins and enzymes has yet to be established. In gastric cancer, the Pl3K/Akt cell regulation pathway has been hypothesised to regulate MMP activity and AQP3 expression, and to control a range of normal cellular functions (proliferation, differentiation, metabolism and motility); however, a direct link requires further study.[132] Studies of the blood-brain barrier (BBB) function in both glioblastoma and subarachnoid haemorrhage have suggested that BBB breakdown products from MMP-3 and MMP-9 have a key role in the function of AQP4.[115] When AQP4 fails to maintain normal water homeostasis across the BBB, cerebral oedema is the result; however, a precise mechanism for how AQP4 fails to function and how MMPs play a role in this has yet to be established. Multiple other studies have also made the link between AQP4, MMP-9 and cerebral oedema, and work continues in this area.[285-288] Further elucidation of the AQP–MMP interaction may reveal a more effective or better-tolerated pharmacological therapy.

4. 2. 5 Immunological sub-types of CRS

When studying the immune response present in the sinonasal mucosa of CRS patients, multiple sub-types of inflammatory patterns have been identified. However, no satisfactory subdivision of disease phenotypes has to date been shown to match

immunological disease status.[242, 289] Initial studies in Caucasian populations suggested CRSwNP was associated with eosinophilic T_{H2}-mediated inflammation, while CRSsNP was associated with a neutrophilic T_{H1}-mediated response.[243] Ethnic differences in inflammatory polyp immune status were subsequently identified, challenging the hypothesis. More recent Asian studies showed a predominance of neutrophilic polyp disease.[290] Significant overlap of inflammatory mediators that regulate T helper cell lineages has been identified and investigation in this area continues.

4. 2. 6 Allergy and immunosuppression

Allergy patients report higher rates of CRS than the general population; however, a pathogenic role for allergy in the development of CRS has yet to be clearly established.[291, 292] Asthma and allergic rhinitis have been strongly associated with CRS in many studies and a significant link has been drawn between these inflammatory conditions of the airway, otherwise referred to as the "united airway theory".[293, 294] The key factors that suggest a link are: common histological features of upper and lower airways; a systemic inflammatory response present in all conditions; and a response of all conditions to the same medication classes. A significant limitation is the embryological origin of the tissues: both upper and lower airways are lined with pseudostratified ciliated columnar epithelium; however, sinonasal tissue is ectodermal while the lower airway epithelium arises from endoderm.[117, 164]

Other immunosuppressive conditions such as HIV, immunoglobin deficiencies, malignancies, oncological treatments and organ transplantation are all associated with severe and refractory sinusitis.[256] These patients are susceptible to CRS complicated by fungal and atypical pathogens.

4. 2. 7 Infection and its role in CRS

Many pathogens have been implicated in CRS aetiology; however, the role of infection becomes less clear as the understanding of CRS pathophysiology improves.[220, 256, 295-297] Early culture studies of CRS sinuses identified bacterial (*Streptococcus pneumoniae, Haemophilus influenza* and *Moraxella catarrhalis*) and fungal pathogens (the *Aspergillus* species).[297] Many organisms are now understood to reside on sinonasal tissue in normal conditions, and recent studies of control

patients identified comparable incidence of these common upper respiratory tract organisms previously implicated in CRS.[295, 298, 299]

4. 2. 8 Biofilms

Biofilms are a structured community of micro-organisms embedded in an extracellular matrix.[300] In CRS, both bacterial and fungal biofilms have been identified as adhering to the sinonasal epithelial surface.[299, 301] In contrast to acute infection by a "planktonic" or freely mobile pathogen, formation of a biofilm represents chronic colonisation of an extracellular matrix which is contained within a capsule of extracellular polymeric substance by the pathogen.[296] The pathogens within the biofilm display structural heterogeneity and genetic diversity. They are safeguarded from host defences by the protective, adherent matrix. It is hypothesised that attempted phagocytosis as part of the host's defence is ineffective toward the adherent pathogen community but does result in localised damage to the host epithelium, leaving the surface further susceptible to inflammatory response and repeated acute infection by the pathogen.[299]

4. 2. 9 Staphylococcus aureus superantigens

The role of *Staphylococcus aureus* as a bacterial colonising agent of the nose is well established with approximately 20–30% of the general population found to be persistent carriers.[302] While *S. aureus* is frequently identified in acute and chronic rhinosinusitis studies, its clear disease-modifying role had not been established until the identification of IgE antibodies to *S. aureus* enterotoxins A and B.[303] Intracellular reservoirs of the bacteria have recently been identified in nasal epithelia amongst other cell types, with this confirmed by peptide nucleic acid fluorescence *in situ* hybridisation (PNA-FISH).[298]

S. aureus is also able to activate B and T lymphocytes independent of any host adaptive immune response. The bacteria directly bridge with the MHC class II molecules on T-cell receptors without requiring processing by an antigen-presenting cell.[304] This characteristic has given rise to the term "superantigens". While direct causality of CRS by biofilms has not been determined, *S. aureus* superantigens have been strongly associated with eosinophilic inflammatory processes including nasal polyps, asthma and aspirin-exacerbated respiratory disease, as well as increased concentrations of cytokines IgE, eosinophil cationic protein (ECP), eotaxin and IL-5, all of which are features of some CRS sub-types.[241]

4. 2. 10 Anatomical variation and mechanical blockage of the upper airway

Finally, anatomical variation of the upper airway, sinuses and the ASL drainage pathways has a role to play in CRS development, predisposing some patients to mucus stasis and prolonged mucosal pathogen exposure.[305, 306] The nasal septum, turbinates, osteomeatal unit and pneumatisation of the sinuses vary between individuals, and injury or obstruction of these areas can affect normal defence mechanisms in the region. Oedema or thick mucus leads to mechanical obstruction of the mucus drainage pathways and contributes to mucus stasis.[307, 308] Blockage of the osteomeatal complex is likely to affect the maxillary, ethmoid and frontal sinuses and is the most common target site for sinus surgery. Improving drainage of the sinuses at these locations is the goal of surgery and usually results in symptomatic improvement for patients.[220]

Chapter 5. AQPs in CRS

5. 1 Aquaporins in chronic rhinosinusitis

5. 1. 1 A role for aquaporins in CRS pathogenesis?

Ultimately, the aetiology of CRS remains unknown. It is understood to be a host vs. environment condition with the important factors in CRS development described in the previous chapter. Host factors such as allergy, altered immune response, epithelial dysfunction and the described histological features of CRS all contribute, as do exogenous factors such as infective pathogens, biofilms and superantigens.[220] We identified alteration of water transport as a possible common link between several of the pathological features of CRS: mucosal oedema, thickened secretions, tissue remodelling and polyp formation. Other examples of conditions where altered water transport or AQP expression and/or function can be compared to CRS have been described in this literature review. In cerebral oedema formation, AQP4 expression and localisation plays a key role (Section 1.9.1). In Sjogren's syndrome, altered AQP5 expression has been implicated in pathological thickened secretion production (Section 1.9.2). AQPs also have an established role in tissue remodelling (Section 1.7.1). Future investigation of the role of altered water transport in any or all of these features in CRS requires an improved understanding of normal water transport in sinonasal tissue, and of sinonasal AQP expression and function.

5. 1. 2 Previous studies of AQPs in CRS

Only a small group of published studies have directly investigated AQP expression in CRS or nasal polyps.[213-215]

The first study published was by Altuntas et al.: they used immunohistochemistry to investigate AQP1 and AQP4 expression of AQP1 in CRSwNP polyp tissue. [215] They compared the normal middle turbinate tissue of 10 patients to polyps from 34 CRS patients. AQP1 was strongly expressed in polyp fibroblasts, glandular epithelium (apical or basolateral expression was not specified) and vascular endothelium. AQP4 was not successfully identified in any of the study samples, and no other AQPs were investigated.

A more recent study by Seno et al. used semi-quantitative PCR to investigate AQP1, AQP3 and AQP5 in normal controls (discussed in Section 3.2.1) and polyp tissue from CRS patients.[213] Their study did not identify any statistically significant difference in AQP mRNA expression between groups. This result is in contrast to the

Altuntas study; however, different methodology was used and this study was not as highly powered.

Shikani et al. investigated the expression of AQP5 using semi-quantitative PCR and immunohistochemistry.[214] They used sphenoid or ethmoid sinus mucosa of five normal patients, and seven CRSsNP and seven CRSwNP patients. They found no difference in AQP5 protein localisation between the groups; however, there was approximately 10-fold less AQP5 mRNA in the CRSwNP specimens, which contrasted with the findings by Seno et al.

Several Chinese studies have investigated AQP1, AQP2 and AQP5 expression in human nasal polyps[309-313]. However, the data should be interpreted with some caution due to previously noted eosinophilic vs. neutrophilic differences between nasal polyps of different ethnic groups.[263, 264] Guan et al. identified increased AQP1 expression in nasal polyp surface and glandular epithelium when compared to normal turbinate tissue using immunohistochemistry.[309] Zhang et al. identified higher AQP2 localisation in polyp epithelium when compared to normal turbinate tissue, also using immunohistochemistry.[310] Using immunohistochemistry, Li et al. found lower localisation of AQP5 in polyp epithelium when compared to normal turbinate tissue: in addition, they were the only group in the literature to date to report AQP5 expression in the endothelium of vasculature.[311] Huang et al. compared AQP5 expression in polyp and normal turbinate using qRT-PCR and Western blot analysis, identifying higher AQP5 mRNA expression in the turbinate tissue, but were not able to confirm this finding using Western blot analysis.[313] Jiang et al. examined AQP5 in different types of nasal polyps, including tissue from patients with and without allergic rhinitis.[312] AQP5 was localised to the surface and glandular epithelia in both groups, with stronger labelling in the allergic rhinitis tissue.

One other Chinese study also looked at the effect of corticosteroid use on AQP expression.[314] Sinonasal tissue was harvested from rat models of allergic rhinitis and normal controls after treatment for one month with either an intranasal corticosteroid or an H1-receptor antagonist. AQP5 was found to be lower in the corticosteroid group when compared to the group with the other treatment and to an untreated allergic rhinitis control group, suggesting that corticosteroid may play a role in down-regulation of AQP5 expression in allergic rhinitis. To date, no published studies have assessed the effect of any other medications on AQP expression in CRS or polyps.

In summary, two studies have identified differences in expression of AQPs in CRS sinonasal tissue: higher AQP1 protein in CRSwNP compared to normal tissue[215] and lower AQP5 mRNA in CRSwNP tissue.[214] The Shikani study's findings did not correlate with the initial findings[213]leaving a role for higher-powered investigations to clarify the situation. Other studies looking at polyp tissue identified several differences in AQP expression when compared to normal sinonasal tissue; however, the disease groups were varied and the power of the studies was not reported, limiting the ability to interpret the significance of these findings.

5. 2 Hypothesis development background

Overall, while some preliminary studies of AQP expression in the sinonasal mucosa of CRS patients or polyps have been performed, this field of research has many avenues left to explore. Some study of AQP1, AQP2, AQP3, AQP4 and AQP5 has been performed; however, most other human AQPs remain uninvestigated. While interesting findings have been produced in this field, a comprehensive study looking at AQP expression in normal sinonasal tissue or sinus disease has yet to be performed. The exploration of AQP expression in normal sinonasal tissue will improve our understanding of normal sinonasal homeostasis. It may provide insight into the aetiology of and treatments for sinonasal disease. Based on this review of the literature, it is clear that anatomical and histological knowledge of the sinonasal region is extensive; however, the understanding of normal sinonasal physiology and the pathophysiology of sinonasal disease remains incomplete.

Distinct physiological roles between the nose and lower airways exist in the filtration and humidification of inhaled air, in comparison to gas exchange at the alveolus and mucus reabsorption in larger airways. While there are some physiological similarities between the upper and lower respiratory tracts (in particular, the "united airway" concept for chronic inflammation), there are also significant differences in the tissue origin and function of the two types of airway. Therefore, research findings on AQP function and localisation in one region should not automatically be extrapolated to the other. Despite the expression profile of some AQPs in sinonasal mucosa being consistent with that observed in the lower respiratory tract, a specific study of sinonasal tissue is critical to our understanding of sinonasal AQPs in health and disease. The literature does not include a comprehensive study of all 13 mammalian AQPs in sinonasal mucosa. Furthermore, no study has explored normal physiological

function of sinonasal AQPs, let alone alteration in sinus disease. Review of the available literature has established a rationale for our investigation of AQP expression in normal sinonasal tissue and in CRS.

Chapter 6. Hypothesis

6. 1 Hypothesis

We hypothesise that human AQPs are expressed in normal sinonasal mucosa, and that there is a difference in AQP expression profiles when CRSwNP and CRSsNP tissues are compared to normal tissue.

6. 1. 1 Research statements to be tested

- Human AQP0–AQP12b mRNA is expressed in normal sinonasal tissue
- AQP mRNA expression is altered in CRSsNP and CRSwNP when compared to normal sinonasal mucosa
- Human AQP1, AQP3, AQP4, AQP5, AQP7, AQP10 and AQP11 proteins are expressed in normal sinonasal tissue
- Localisation of these AQPs is altered in CRSsNP and CRSwNP when compared to normal sinonasal mucosa

Chapter 7. Materials and Methods

7. 1 Ethics

The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee as a part of application number 19.08 “The Innate Immune System in Chronic Rhinosinusitis”.

7. 2 Tissue samples

Flinders ENT has a sinus tissue bank. Tissue was collected from patients following their informed consent and stored in accordance with ethically approved university protocols.[315] Sinus tissue samples were routinely taken from the ethmoidal sinus infundibulum.

7. 2. 1 Normal sinonasal tissue

Normal sinonasal mucosa was used as the experimental control tissue. This was obtained from patients undergoing endoscopic trans-sphenoidal surgery, who had no history or radiological evidence of sinus disease and a clinically normal mucosal appearance at the time of surgery.

7. 2. 2 Patients with CRS

Patients with a diagnosis of CRS who had failed medical management and had consented to undergo functional endoscopic sinus surgery (FESS) were recruited to the study. Patients undergoing revision surgery or FESS for a clinical indication other than CRS, and patients with significant co-morbidities (e.g. cystic fibrosis) were excluded. Each patient was investigated and the following data were recorded: sex; age at surgery; clinical examination findings at pre-operative consultation (i.e. presence of polyps); and a computed tomography (CT) report confirming sinus disease; and all patients were assigned a Lund–MacKay score (severity of sinus disease on the CT scan).[316] Every operation report was reviewed to confirm the following: the type of surgery, presence of polyps and presence of mucin. Based on the collated data, patients were assigned to disease groups CRSsNP and CRSwNP as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps [EPOS] 2012.[220]

7. 2. 3 Tissue collection

Following tissue harvest, two samples from each patient were stored. Samples for RNA analysis were stored in RNAlater™ (Ambion, USA) at -20°C prior to processing. Samples for immunohistochemical (IHC) analysis were placed in 10% buffered formalin prior to preparation into paraffin blocks.

7. 3 Polymerase chain reaction (PCR) experiments

7. 3. 1 RNA extraction

Previously extracted RNA, frozen at -80°C, was available for 25 patients from the tissue bank who fulfilled the inclusion criteria for this study. The RNA extraction was performed as described in Woods et al.[315] For unprocessed sinonasal tissue samples (n=10), up to 0.025 grams (Shimadzu® balance) were homogenised (Dremmel® "MultiPro™ 10,000-37,000 RPM" drill) in 1 ml of TRIzol® (Invitrogen Life Technologies, NY, USA). A modified RNA extraction was then performed, as previously described by Woods et al.[315] The TRIzol® extraction protocol was modified to include a high salt precipitation step recommended by the manufacturer in tissues with proteoglycans and/or polysaccharides. The resultant RNA pellet was dissolved in 22 µl ultra-pure water (UPW).

7. 3. 2 Spectrophotometry

The RNA concentration of all RNA samples (n=35) was determined using the NanoDrop 8000 spectrophotometer (ThermoScientific, Wilmington, Delaware, USA) prior to RNA quality control using the Agilent® 2100 Bioanalyzer™ (Agilent Technologies, California, USA). Samples with inadequate RNA were excluded from further analysis after review of the spectrophotometry results (n=3).
The exclusion criteria were:

- degraded RNA (confirmed by review of digital gel electrophoresis image produced by Bioanalyzer software)
- RNA integrity number found to be <4.0[317]
- very low concentration of RNA in sample.

The remaining patient samples (n=32) included in the study were: control sinonasal tissue (n=9), CRSsNP tissue (n=10) and CRSwNP tissue (n=13).

7. 3. 3 RNAse treatment and cDNA synthesis

A single-step elimination of contaminating genomic DNA and reverse transcription (RT) was performed using the RT^2 First Strand™ kit (Qiagen, Venlo, Netherlands). The RNA was diluted to 125 ng/µL ultra-pure water (UPW); then, 1 µg of RNA was added to 2 µL of buffer and 10 µL of genomic DNA elimination mix. The solution was incubated at 42°C for five minutes before cooling on ice for one minute.
Two negative controls were selected from each patient group prior to cDNA synthesis. Reverse transcriptase was omitted from the cDNA synthesis process for these negative reverse transcription (RT) controls. An additional ultra-pure water (UPW) reverse transcription (RT) negative control was included during the cDNA synthesis process.
The cDNA was diluted to 1:15 in ultra-pure water (UPW), parafilmed in sealed tubes and stored at 4°C prior to the polymerase chain reaction (PCR).

7. 3. 4 Real-time quantitative polymerase chain reaction (PCR)

Each cDNA sample was diluted with ultra-pure water (UPW) to a total volume of 500 µL prior to being assayed in triplicate using RT^2 SYBR Green ROX FAST master mix (Qiagen, Venlo, Netherlands). A genomic DNA positive control and an ultra-pure water (UPW) negative PCR control were included for each assay. Commercial RT^2 qPCR Primer™ assays (Qiagen, Venlo, Netherlands) were used to assess the mRNA gene expression of the 13 AQP genes (Table 7.1). The final reaction volume was 25 µL which comprised: 12.5 µL of SYBR Green master mix; 1 µL of qPCR primer assay; 1.5 µL of ultra-pure water (UPW); and 10 µL of sample cDNA. Aquaporin (AQP) PCR conditions consisted of initial enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 30 seconds, then a final hold from 65°C to 95°C for melt curve analysis in the Rotor-Gene™ 6000 cycler (Corbett Research, Australia). High resolution melts were also performed, using a melt range from 71°C–98°C. Individual melt ranges were selected for each assay based on the initial melt temperature of the assay product.

Table 7.1: Commercial quantitative PCR primer assays for human AQPs

Target gene	Entrez Gene ID	PCR product band size	Catalogue no. (commercial assays)
Aquaporins			
AQP0 (MIP)	36456	103 bp	PPD04546A
AQP1	358	132 bp	PPH59997A
AQP2	359	101 bp	PPH00017A
AQP3	360	115 bp	PPH14747A
AQP4	361	111 bp	PPH05944B
AQP5	362	106 bp	PPH16382A
AQP6	363	70 bp	PPH09464A
AQP7	364	88 bp	PPH22164B
AQP8	343	146 bp	PPH13652A
AQP9	366	91 bp	PPH05945A
AQP10	89872	83 bp	PPH15277A
AQP11	282679	153 bp	PPH16785A
AQP12b	285192	86 bp	PPH60610A
Housekeeping genes			
18S rRNA	100008588	100 bp	PPH05666E
HPRT	3251	57 bp	PPH01018C

Table 7.1: The 13 commercial RT2 qPCR Primer™ AQP assays used in our experiment are presented. Also listed are assays for the two housekeeping genes: 18S rRNA (human 18S ribosomal RNA) and HPRT (hypoxanthine phosphoribosyl transferase) (Qiagen, Venlo, Netherlands).

7. 3. 5 Housekeeping gene selection

In the absence of a universal control gene for sinus tissue, seven housekeeping genes were assessed for normalisation: 18S rRNA, HPRT, ubiquitin C (UBC), beta-actin, porphobilinogen deaminase (PBGD), glyceraldehyde-3-phosphate dehydrogenase (GAPD) and L32 (ribosomal protein L32). Commercial RT^2 qPCR Primer™ assays were used to assess 18S rRNA and HPRT with these prepared as described above. The protocol and PCR conditions used for the additional five primers listed in Table 7.2 had been previously determined in the Upper Gastrointestinal (GI) Disease Research Laboratory at Flinders Medical Centre.[318] The final reaction volume was 20 µL: 10 µL of 2x QuantiTect SYBR Green master mix; 2 µL forward gene-specific primer; 2 µL reverse gene-specific primer (final concentration of each primer in PCR reaction=250 nM); and 6 µL of cDNA sample. The results were normalised using the method outlined below.

Table 7.2: Additional quantitative PCR primer assays for housekeeping genes

Target gene	Entrez Gene ID	PCR product band size	Primers	
UBC	7316	119 bp	Forward:	5'-ATT-TGG-GTC-GCG-GTT-CTTG-3'
			Reverse:	5'-TGC-CTT-GAC-ATT-CTC-GAT-GGT-3'
Beta-Actin	60	174 bp	Forward:	5'-GCC-GAT-CCA-CAC-GGA-GTA-CT-3'
			Reverse:	5'-TTG-CCG-ACA-CGA-TGC-AGA-AG-3'
PBGD	811149	145 bp	Forward:	5'-CTT-TCC-AAG-CGG-AGC-CAT-GTC-TGG-3'
			Reverse:	5'-CAT-GAG-GGT-TTT-CCC-GCT-TGC-AGA-3'
GAPD	2597	130 bp	Forward:	5'-TGC-ACC-ACC-AAC-TGC-TTA-GC-3'
			Reverse:	5'-GGC-ATG-GAC-TGT-GGT-CAT-GAG-3'
L32	6161	147 bp	Forward:	5'-TTC-CTG-GTC-CAC-AAC-GTC-AAG-3'
			Reverse:	5'-TTG-TGA-GCG-ATC-TCG-GCAC-3'

Table 7.2: Housekeeping genes: UBC (ubiquitin C); beta-actin; porphobilinogen deaminase (PBGD); glyceraldehyde-3-phosphate dehydrogenase (GAPD); and ribosomal protein L32 (L32).

7. 4 Verification of PCR products

The PCR products from each of the housekeeping gene and AQP assays were assessed using the Rotor-Gene™ software package. The amplification take-off cycle and triplicate melt profiles for each sample were assessed: the assay product that failed to take off and those with sporadic amplifications other than the assay target were excluded. Results for all samples were compared to the negative and ultra-pure water (UPW) controls to confirm the consistent production of PCR product by each assay. A melt curve analysis was performed at the end of each PCR assay. Single melting point peaks were identified for the target and reference housekeeping genes.

The melt curves from sinus samples were referenced against the melt curves from control gDNA to ensure amplification of the appropriate sequence.
For the commercial PCR assays, experimental data were cross-referenced with the manufacturer to ensure consistent gDNA control melt curves with their reference DNA sequence-verified products (the results are shown in Table 8.2). For the housekeeping genes previously worked up in the Flinders Medical Centre Upper Gastrointestinal (GI) Disease Research Laboratory, corresponding melt curve and DNA sequence verification data were available to allow confirmation that the correct product was amplified. Relative expression values for each gene for each sample were derived from the real-time qRT-PCR data using Q-Gene software.[319]

7. 5 Normalisation of real-time quantitative PCR data

In the absence of a universal control gene for sinus tissue, the geometric mean of multiple human housekeeping (HK) genes was used for normalisation. [320] The GeNorm™ algorithm was used to select the three most stably expressed housekeeping genes observed in the sinus tissue samples.[320] This was useful to control for bias introduced by single-control normalisation and more accurate where the difference in expression of the genes of interest is likely to be subtle. The GeNorm™ algorithm employed by RealTime StatMiner™ (Integromics Software) calculated the average pairwise variation in expression of each housekeeping gene with the other control genes across all tissue samples, giving a gene stability score, or M value. A lower M value correlates to increasingly stable gene expression in the tissue analysed.
The three most stable housekeeping genes identified in the sinus tissue specimens were identified (Table 8.3), and the geometric mean of the relative expression of these three housekeeping genes was employed as the normalisation factor.

7. 6 PCR data statistical analysis

The normalised relative concentration data for each AQP were analysed using PRISM version 6.0c (GraphPad Software, Inc). The distribution of data points was tested using the Shapiro–Wilk test (Table 8.4). The majority of the data points across each AQP were not normally distributed so non-parametric analysis was chosen.
To determine a difference in AQP expression between the groups, normal vs. CRSwNP vs. CRSsNP, analysis of variance (ANOVA) was performed for non-parametric data (Kruskal–Wallis analysis) with $p<0.05$ considered statistically

significant. Dunn's test for multiple comparisons was then applied for further sub-group analyses of control vs. CRSwNP and control vs. CRSsNP. The results were graphed using the PRISM package.

7. 7 Immunohistochemistry

7. 7. 1 Tissue samples

The tissue samples included in the immunohistochemistry study were: control (n=5), CRSsNP (n=10) and CRSwNP (n=13). There were fewer control tissues than tissues analysed with PCR because five control samples had insufficient tissue for analysis. Routine haematoxylin and eosin (H&E) stains were performed by SA Pathology staff on a section of all samples at the time of storage. Immunohistochemistry (IHC) slides were prepared from the paraffin-blocked samples using protocols previously established in the Flinders University Anatomical Pathology Department for the study of AQPs in the lower respiratory tract.[130, 321]

7. 7. 2 Selection of target AQPs

Aquaporins (AQPs) known to be expressed in the respiratory tract were selected for immunohistochemical (IHC) study in sinonasal mucosa (AQP1, AQP3, AQP4 and AQP5). On the basis of the PCR results, several additional AQPs of interest were also identified (AQP7, AQP10 and AQP11).

7. 7. 3 Antibody selection

Antibodies used in this study and corresponding retrieval techniques are presented in Table 7.3 and Table 7.4. Commercial anti-AQP antibodies optimised in previous departmental studies of the respiratory tract were used for AQP1, AQP3, AQP4 and AQP5. Commercially available antibodies for AQP7, AQP10 and AQP11 were identified from the literature on AQP expression in other anatomical locations. Conditions for each anti-AQP antibody were optimised using individual positive control tissues (human) for each AQP: lung for AQP1 and AQP5, and kidney for AQP3 and AQP4. Based on published reference photos, positive control tissues were selected for optimisation of the additional antibodies: testis for AQP7[94]; jejunum for AQP10[108]; and kidney for AQP11.[62] After extensive optimisation attempts, it was found that the anti-AQP 10 antibody did not label any AQP10 in either control intestinal tissue or sinus tissue and therefore investigation of this AQP was not pursued further.

Table 7.3: Anti-aquaporin primary antibodies for immunohistochemical study

Aquaporin	Antibody info	Dilution	Company	Retrieval
AQP1	Rabbit anti-human, polyclonal	1:12000	Alpha diagnostics	No retrieval
AQP3	Rabbit anti-human, polyclonal	1:5000	Millipore diagnostics	Citric acid
AQP4	Mouse, monoclonal	1:500	AbD Serotec	Trypsin
AQP5	Rabbit, monoclonal	1:1000	Epitomics	Alkaline
AQP7	Rabbit anti-rat/human, polyclonal	1:100	Alpha diagnostics	Alkaline
AQP10	Rabbit anti-rat, polyclonal	Not found	Alpha diagnostics	N/A
AQP11	Rabbit anti-mouse, polyclonal	1:200	Alpha diagnostics	Trypsin

Table 7.4: Antigen retrieval techniques

Retrieval	Technique
Citric acid	De-waxed samples were immersed in 0.01 M citrate buffer, pH 6.0, heated to boil for three minutes using Samsung "The Timesaver II"© microwave "normal" setting, then maintained at the boil for 10 minutes on "low". Samples were then cooled for one hour to room temperature before 10% normal goat serum was applied.
Trypsin	A 500-watt water bath was used to heat 400 mLs tris buffered saline to 37°C for 30 minutes, then 0.4 g $CaCl_2$ (calcium chloride dihydrate AnalaR®, Merck) and 0.4 g trypsin (Difco™ Trypsin 250, BD Biosciences) were added before heating for 10 minutes. De-waxed samples were immersed in this solution for 30 minutes at 37°C then rinsed with water and TBS before 10% normal goat serum was applied.
Alkaline	De-waxed samples were immersed in Dako alkaline target retrieval solution, pH 9.0, heated to boil for three minutes using Samsung "The Timesaver II"© microwave "normal" setting, then maintained at the boil for 10 minutes on "low". Samples were then cooled for 30 minutes to room temperature before 10% normal goat serum was applied.

7. 8 Immunohistochemical (IHC) labelling protocol

To facilitate the cutting of bony tissue segments, all sinus samples were decalcified by immersing the face of the paraffin tissue block in a solution containing 1% ethylenediaminetetraacetic acid (EDTA) and 7% hydrochloric acid (HCl) for 15 minutes then rinsed well in distilled water (dH_2O). Paraffin sections of each sample were cut 4 µm (micrometres) thick, mounted onto glass slides, de-paraffinised and rehydrated before quenching with 1% hydrogen peroxide in 50% ethanol. The sections were washed with a TBS (tris/salt) solution, pH 7.6, and applicable antigen retrieval was performed. Anti-AQP antibodies were diluted to required concentrations (Table 7.3) with a non-specific antibody diluent (10% normal goat serum, Sigma, G9023-10mL): 200 µL of antibody was applied to each section and incubated overnight at 4°C. The DakoCytomation EnVision™ Dual Link detection system (Dako, Denmark) was used in accordance with the manufacturer's protocol, then sections were counterstained with haematoxalin and lithium carbonate before washing and dehydration using xylene rinses. Coverslips were applied using DePeX (VWR international, Pennsylvania, USA).
The inclusion of positive and negative control samples confirmed the function of the anti-AQP antibodies and detection system. Positive control slides for each anti-AQP antibody were cross-referenced against images in the published literature to confirm that the labelling of the target AQP was as expected in the reference tissues: AQP1 and AQP5 in lung; AQP3, AQP4 and AQP11 in kidney; and AQP7 in testis. [62, 67, 82, 83, 94, 108, 128, 213, 215, 322-325] Negative controls were also cross-referenced to ensure that the detection system did not label any protein or structure in addition to the target AQP (see Appendix: Immunohistochemistry controls).

7. 9 Scoring of tissue samples

Slides were viewed on an Olympus BX 50 Brightfield Photomicroscope™, Q-Imaging MicroPublisher™ RTV 5 megapixel digital camera and Q-Capture software© (Q-Imaging, Canada). A semi-quantitative 6-point Likert scoring scale was used to assess the intensity of antibody labelling (-, +/-, +, ++, +++ or "not present") for each of a list of histological sub-sites (Table 7.5). Samples were excluded from the statistical analysis for that sub-site if they had a score of "not present".

The listed sub-sites were selected for examination in order to gain a better understanding of AQP distribution throughout sinonasal tissue, to determine if this was similar to other parts of the respiratory tract and to direct future study of the role of AQPs in normal function to these areas. Different AQPs are known to play discrete functional roles in different parts of the respiratory tract and at different tissue sub-sites (as described in the literature review in Section 3.1: AQPs in the respiratory tract). Based on the fact that CRS is primarily a disease of altered host vs. environment response at the sinonasal epithelium, detailed investigation of this area was undertaken, including assessment of AQP localisation to apical vs. basolateral vs. cytoplasmic sites within the epithelial cells. Vasculature was defined as any vessels visible within the tissue section. The connective tissue category included all tissue not included in another category. After discussion with the senior pathologist regarding the IHC methodology, it was concluded that if significant findings were identified within this connective tissue, further cellular-level characterisation of cells and structures could be performed.

Table 7.5: Sinonasal tissue scoring sites and sub-sites

Area	Sub-site
Surface Epithelium	Apical
	Basolateral
	Cytoplasmic
Glandular Epithelium	Apical
	Basolateral
	Cytoplasmic
Vasculature	-
Connective tissue	-

All tissue sample sections were inspected and scored as a series, that is, the haematoxalin and eosin (H&E) slide and then each of the six AQPs. The disease group allocation was not apparent to the scorer while carrying out the scoring as samples were labelled with an identification code only. Areas of tissue demonstrating salient findings were photographed for reference (images were processed using ImageJ™, US National Institutes of Health). The H&E slide was used in each case to

identify the relevant structures before comparison with the AQP slides for the scoring of AQP expression intensity. For each tissue sample, the set of slides including each AQP assessed were compared to confirm that examination and scoring were undertaken in consistent areas of the same tissue.

7. 10 IHC statistical analysis

Raw scores for each sample sub-site were imported into the GraphPad PRISM® 6.0 software package, collated by disease group and a median for each group was calculated (a score of "not present" or 0 was excluded from analysis). To determine the difference in AQP localisation intensity between groups: normal vs. CRSwNP vs. CRSsNP, ANOVA analysis was performed for non-parametric data (Kruskal–Wallis analysis): $p<0.05$ was considered statistically significant. Dunn's test for multiple comparisons was then applied for sub-group analyses: control vs. CRSwNP and control vs. CRSsNP. Results were graphed using the PRISM package.

Results

Chapter 8. PCR results

8. 1 Patient demographics

Tissue samples from 32 subjects were included in the PCR study (Table 8.1), 18 males and 14 females. Distribution of sexes across the patient groups was not similar (chi-square p=0.02). A large number of females were in the control group, more male patients than female patients were in the CRSwNP group, and the sexes were approximately even in the CRSsNP group. There was no significant difference between the groups when looking at patients' ages at the time of surgery (Kruskal–Wallis p=0.38). The CRS disease severity as indicated by Lund–Mackay CT scan scores was not significantly different between the CRSsNP and CRSwNP groups (Mann–Whitney U test p=0.13).

Table 8.1: Patient characteristics – PCR tissues

PCR study	Controls (n=9)	CRSwNP (n=13)	CRSsNP (n=10)	Test p-value
Sex (M/F)	3/6	11/2	4/6	Chi-square p=0.02
Mean Age at Surgery	45.4	46.8	38.8	Kruskal–Wallis p=0.38
Lund–Mackay CT Score Mean (range)	N/A	12.8 (6-20)	9.2 (1-20)	Mann–Whitney U test p=0.13

Table 8.1: Demographic data for patients included in the PCR study indicated a statistically significant difference in sex distribution between the groups. Age and disease severity on CT scans were comparable between the groups.

8. 2 Verification of PCR products

The PCR product melt temperatures for each assay are presented in Table 8.2. The high-resolution melt temperature for the product of each assay correlated well to the reference temperature provided by the manufacturer, confirming the assays were functional.

Table 8.2: Assay product high-resolution melt comparison

Aquaporin	Study gDNA assay product melt temp (°C)	Manufacturer's reference melt temp (°C)	Difference between temps (°C)
MIP (AQP0)	80.93	83.4	2.47
AQP1	85.50	87.0	1.50
AQP2	84.27	86.0	1.73
AQP3	82.50	84.6	2.10
AQP4	76.35	79.8	3.45
AQP5	81.05	83.8	2.75
AQP6	79.95	82.4	2.45
AQP7	80.15	82.6	2.45
AQP8	84.90	86.2	1.30
AQP9	75.18	78.5	3.32
AQP10	81.47	83.2	1.73
AQP11	82.72	85.0	2.28
AQP12b	86.95	88.2	1.25

Table 8.2: The table demonstrates confirmation of functional PCR assays supplied by Qiagen. For each AQP assay, a gDNA high resolution melt profile was assessed and compared to the reference melt profile for gDNA provided by the manufacturer.

8. 3 Housekeeping genes for data normalisation

Hypoxanthine phosphoribosyl transferase (HRPT), ubiquitin C (UBC) and beta-actin (B-actin) were identified as the most stable housekeeping genes in the sinus tissue specimens. The geometric mean of the relative expression of these three housekeeping genes was employed as the normalisation factor for expression of the AQP genes in the sample group (Table 8.3).

Table 8.3: M-values – selection of housekeeping genes

Housekeeping Genes	M-value (3 sig figures)
Used for normalisation	
HPRT	**0.480**
UBC	**0.480**
Human B-Actin	**0.539**
Excluded	
18S rRNA	**0.965**
PBGD	**1.14**
GAPD	**0.820**
L32	***0.714***

Table 8.3: M-values for each housekeeping gene were compared and the three genes with the most stable expression (the lowest M-values) were selected.

8. 4 Distribution of data

The distribution of the data was assessed to ensure the appropriate data analysis technique was selected (i.e. was the data normally distributed, and would it require parametric or non-parametric analysis?). Of the 36 results for each individual AQP, 17 were abnormally distributed (Table 8.4). As a result, non-parametric analysis was chosen to interpret the experimental results.

Table 8.4: Normality testing (Shapiro–Wilk test)

AQP	Control	CRSwNP	CRSsNP
AQP0	Abnormal	Normal	Abnormal
AQP1	Normal	Normal	Normal
AQP2	Normal	Abnormal	Normal
AQP3	Normal	Abnormal	Abnormal
AQP4	Abnormal	Normal	Abnormal
AQP5	Abnormal	Normal	Abnormal
AQP6	Normal	Abnormal	Normal
AQP7	Abnormal	Normal	Normal
AQP8	Abnormal	Normal	Abnormal
AQP9	Abnormal	Abnormal	Abnormal
AQP10	Abnormal	Abnormal	Abnormal
AQP11	Normal	Normal	Abnormal

Table 8.4: The normality of the distribution of results from each AQP assay was assessed:

Normal tissue: 5/12 normally distributed results

CRSwNP tissue: 7/12 normally distributed results

CRSsNP tissue: 5/12 normally distributed results

8. 5 AQP mRNA expression in normal sinonasal tissue

The mRNA for 12 of the 13 known mammalian AQPs was successfully identified in normal human sinonasal tissue with a wide range of relative concentrations (Table 8.5, Figure 8.1). The relative concentration of mRNA for several AQPs was extremely low: AQP0, AQP8 and AQP10 were detected with a median relative concentration of <0.01. A low expression with median relative concentration between 0.01–0.12 was seen in AQP2, AQP4, AQP6, AQP7, AQP9 and AQP11. AQP1, AQP3 and AQP5 were found to have high median relative mRNA concentrations with levels between 1.0 and 10.56. No mRNA expression of AQP12b was identified in any sample.

Table 8.5: Relative mRNA concentrations for AQP0–AQP11: normal tissue

AQP	Relative mRNA concentration	
	Median	Inter-quartile range
AQP0	0.00108	0.0005-0.0105
AQP1	3.59	2.03-10.56
AQP2	0.0165	0.001-0.0237
AQP3	1.55	1.551.01-4.92
AQP4	0.12	0.106-0.200
AQP5	2.49	0.797-7.13
AQP6	0.0292	0.0205-0.0397
AQP7	0.028	0.0232-0.0393
AQP8	0.00109	0.000754-0.00446
AQP9	0.0231	0.0160-0.0569
AQP10	0.00683	0.00550-0.00833
AQP11	0.0935	0.0789-0.155

Figure 8.1: Relative mRNA concentrations for AQP0–AQP11: normal tissue

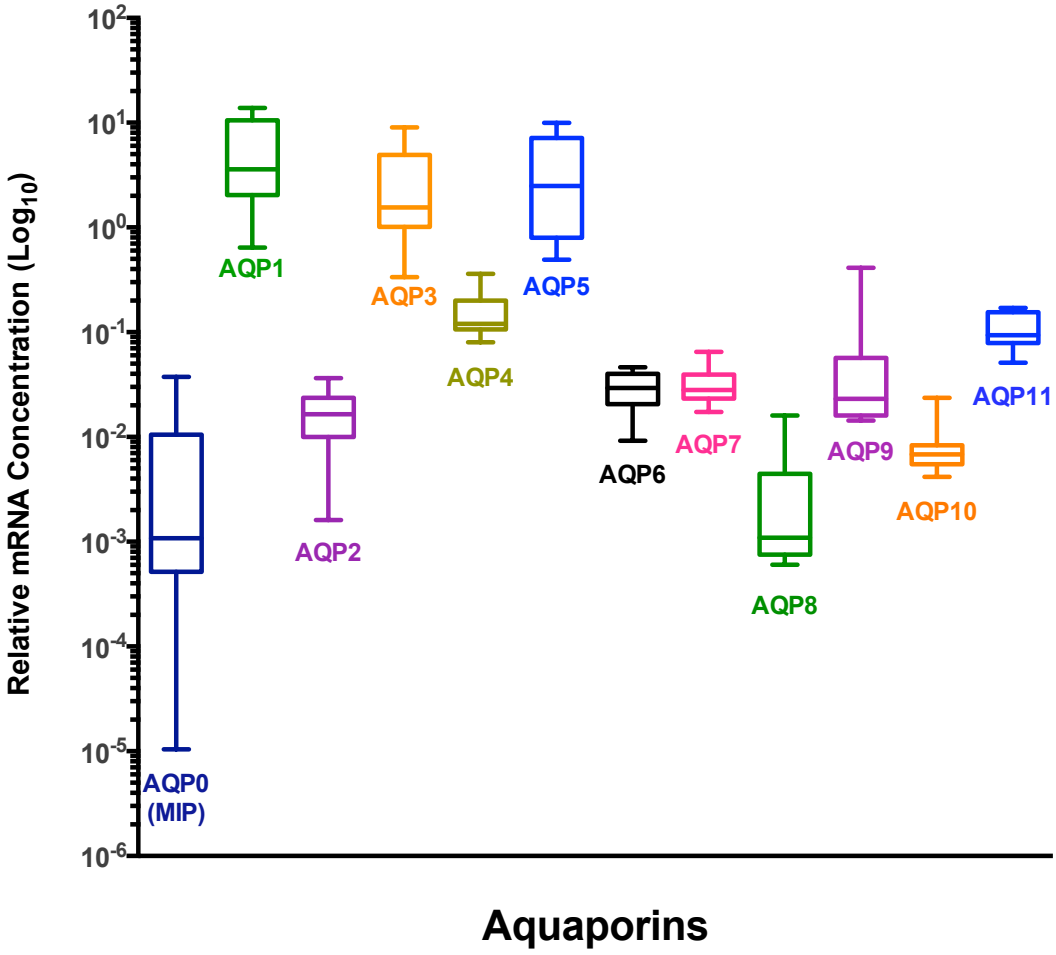

Figure 8.1: The graph demonstrates the relative mRNA concentration of each AQP in normal sinonasal tissue. The data are presented as a box plot with median, interquartile range and range. A logarithmic scale was used due to the wide range of concentrations. No AQP12b mRNA was identified and therefore it has been omitted from the graph.

8. 6 AQP mRNA expression – all groups

The mRNA of AQP0–AQP11 was identified in all tissue samples across all groups (Table 8.6). No mRNA expression of AQP12b was identified in any sample from any group. The analysis of AQP expression comparing normal sinonasal tissue to the two CRS disease groups (Figure 8.2) identified lower mRNA expression of two AQPs: AQP4 was 1.7-fold less in CRSwNP ($p<0.01$) and AQP11 was 1.2-fold less in CRSwNP ($p<0.05$). Differences in mRNA expression approached significance for AQP3 ($p=0.05$), AQP7 ($p=0.07$) and AQP10 ($p=0.09$). The mRNA expression of the

remaining AQPs was not significantly different between control and CRS disease, with or without polyps ($p>0.05$ for all AQPs).

Table 8.6: Relative mRNA concentrations for AQP0–AQP11: all groups

AQP	Relative mRNA concentration			Kruskal-Wallis	Dunn's post-hoc test	
	Control Median (IQR)	CRSwNP Median (IQR)	CRSsNP Median (IQR)	p value	Control vs CRSwNP	Control vs CRSsNP
AQP0	0.00108 (0.0005-0.0105)	0.000886 (0.0003-0.0014)	0.000955 (0.0003-0.0018)	0.6819	N/A	N/A
AQP1	3.59 (2.03-10.56)	4.73 (2.67-7.18)	3.32 (1.89-5.42)	0.4737	N/A	N/A
AQP2	0.0165 (0.001-0.0237)	0.0165 (0.0115-0.0233)	0.0167 (0.0062-0.0203)	0.9488	N/A	N/A
AQP3	1.55 (1.01-4.92)	5.09 (3.42-9.82)	2.54 (1.51-6.75)	0.0519	N/A	N/A
AQP4	0.12 (0.106-0.200)	0.0692 (0.055-0.092)	0.0846 (0.070-0.155)	0.0061	Significant 1.7x down	N/A
AQP5	2.49 (0.797-7.13)	2.1 (1.26-3.25)	3.14 (1.17-5.46)	0.5096	N/A	N/A
AQP6	0.0292 (0.0205-0.0397)	0.0265 (0.0219-0.0339)	0.0296 (0.0169-0.0389)	0.8889	N/A	N/A
AQP7	0.028 (0.0232-0.0393)	0.0175 (0.0140-0.0269)	0.0286 (0.0191-0.0403)	0.0680	N/A	N/A
AQP8	0.00109 (0.000754-0.00446)	0.0018 (0.00108-0.00341)	0.00172 (0.000857-00238)	0.7313	N/A	N/A
AQP9	0.0231 (0.0160-0.0569)	0.0394 (0.0295-0.0625)	0.0299 (0.0217-0.142)	0.5171	N/A	N/A
AQP10	0.00683 (0.00550-0.00833)	0.00286 (0.00236-0.00669)	0.006799 (0.00285-0.0136)	0.0866	N/A	N/A
AQP11	0.0935 (0.0789-0.155)	0.0769 (0.0579-0.0877)	0.0943 (0.0902-0.111)	0.0129	Significant 1.2x down	N/A

Table 8.6: The relative concentration of mRNA for each AQP is presented as the median (interquartile range). The boxes shaded blue indicate that the mRNA expression was statistically significantly different compared to the control ($p<0.05$ Kruskal–Wallis with Dunn's correction for multiple comparisons). N/A indicates a site where statistical significance was not reached with Kruskal–Wallis (KW) testing; therefore, Dunn's correction was not applied.

Figure 8.2: Relative mRNA concentrations for AQP0–AQP11: all groups

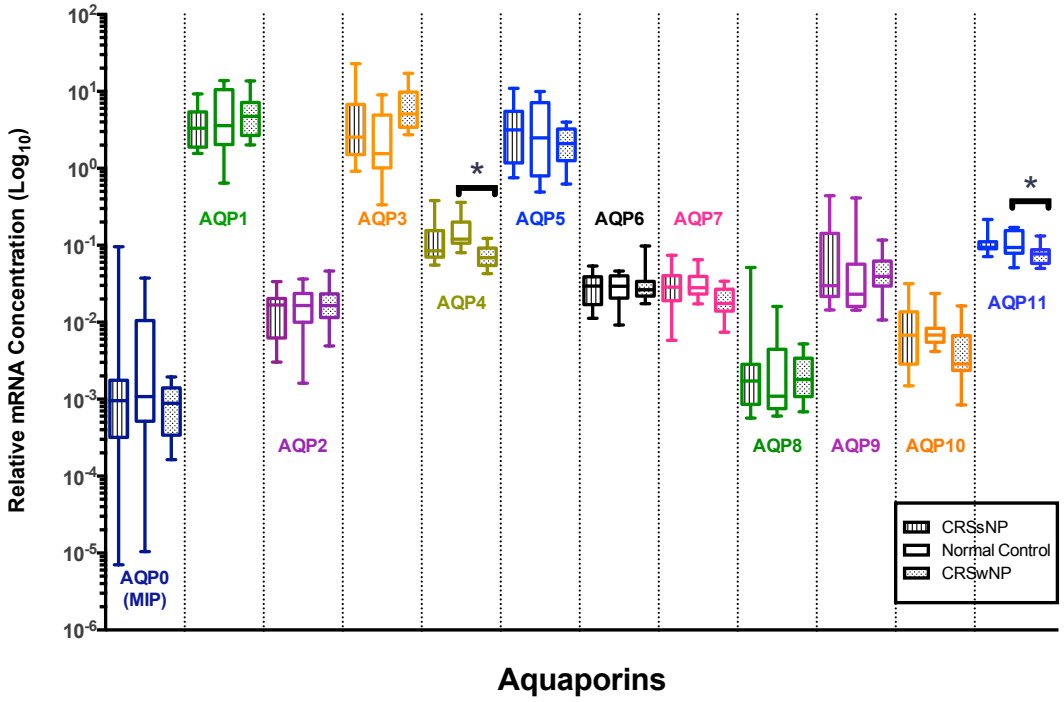

Figure 8.2: The graph demonstrates the relative mRNA concentration of each AQP in each tissue group (CRSsNP, normal control, CRSwNP). The data are presented as a box plot with median, interquartile range and range. A logarithmic scale was used due to the wide range of concentrations. No AQP12b mRNA was identified and it has been omitted from the graph.

**=p<0.05 Kruskal–Wallis with Dunn's correction for multiple comparisons.*

Chapter 9. Immunohistochemistry results

9. 1 Patient demographics

Tissues samples from 28 subjects were included in the immunohistochemical (IHC) study (Table 9.1), 16 males and 12 females. The distribution of the sexes across the patient groups was not similar (p=0.02). The higher number of female patients was statistically significant in the control group, and there were more male patients than female patients in the CRSwNP group. Males and females were approximately even in the CRSsNP group. There was no significant difference in patients' ages at the time of surgery between the groups (Kruskal–Wallis p=0.30), or in disease severity on the CT scan between the CRSwNP and CRSsNP groups (Mann–Whitney U test p=0.13).

Table 9.1: Patient characteristics – Immunohistochemistry tissues

IHC study	Controls (n=5)	CRSwNP (n=13)	CRSsNP (n=10)	Test p-value
Sex (M/F)	1/4	11/2	4/6	Chi-square p=0.02
Age at Surgery (mean)	39.7	46.8	38.8	Kruskal–Wallis p=0.30
Lund–Mackay CT Score (mean) (range)	N/A	12.8 (6-20)	9.2 (1-20)	Mann–Whitney p=0.13

Table 9.1: Demographic data for the patients included in the immunohistochemistry study indicated a statistically significant difference in sex distribution between the groups. Age and disease severity on the CT scan were comparable between groups.

9. 2 Sinonasal tissue architecture

9. 2. 1 Normal tissue

Normal sinonasal mucosa was observed in the control tissue samples with areas of normal pseudostratified ciliated columnar epithelium, submucosal glands, vasculature and connective tissue all identified (Figure 9.1, Figure 9.2).

Figure 9.1: Example of normal sinonasal tissue

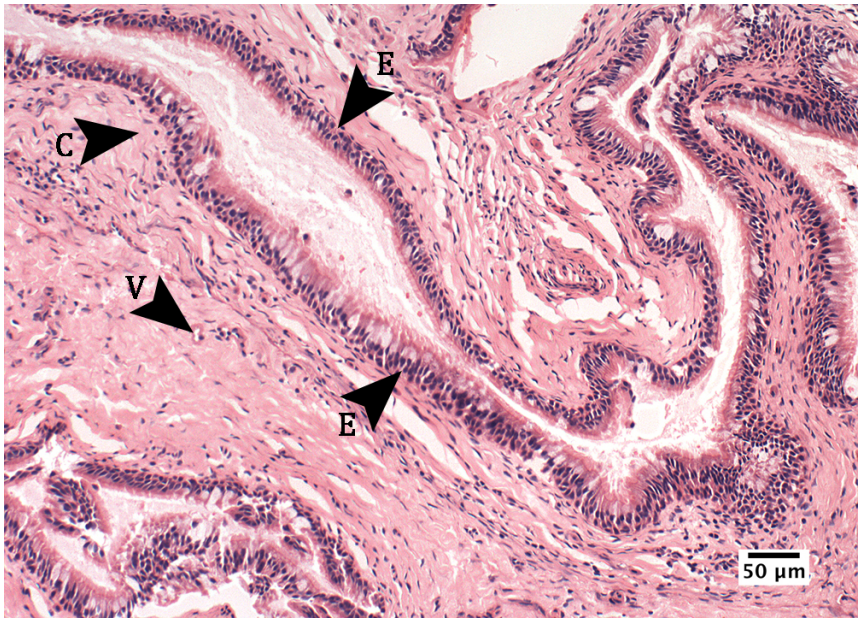

Figure 9.1 and Figure 9.2: These photomicrographs are of normal sinonasal tissue samples (haematoxalin and eosin [H&E] stain). Key structures were identified: (E) pseudostratified ciliated columnar epithelium; (G) submucosal glands; (V) vasculature; and (C) connective tissue. Due to the undulating nature of sinonasal mucosa, multiple layers of epithelium are seen facing each other in the middle of the photomicrograph. There was also some minor separation of the sub-epithelial tissue due to sampling trauma.

Figure 9.2: Example of normal sinonasal tissue

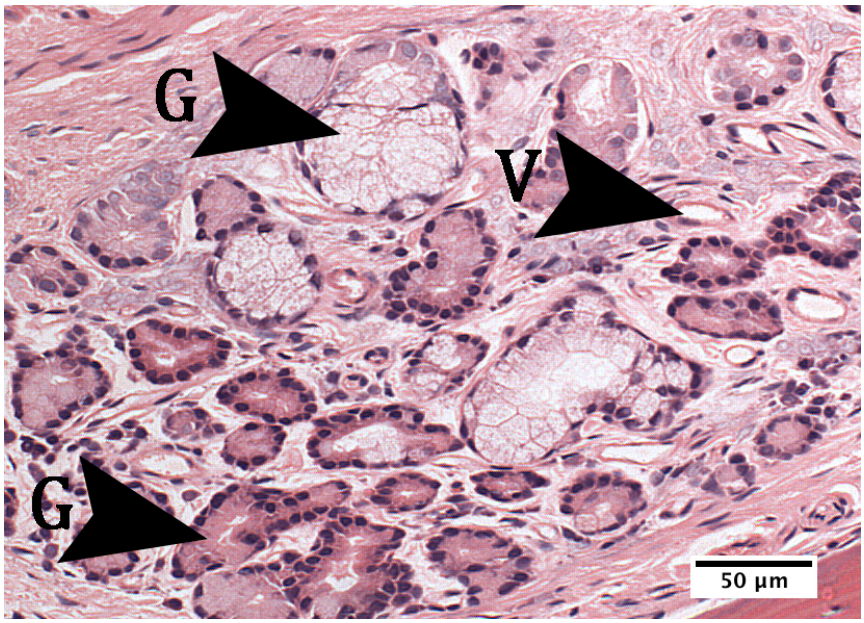

Figure 9.1 and Figure 9.2: These photomicrographs are of normal sinonasal tissue samples (haematoxalin and eosin [H&E] stain). Key structures were identified: (E) pseudostratified ciliated columnar epithelium; (G) submucosal glands; (V) vasculature; and (C) connective tissue. Due to the undulating nature of sinonasal mucosa, multiple layers of epithelium are seen facing each other in the middle of the photomicrograph. There was also some minor separation of the sub-epithelial tissue due to sampling trauma.

9. 2. 2 CRSsNP tissue

Tissue architecture consistent with CRSsNP was observed in the CRSsNP tissue samples analysed (Figure 9.3). Areas of mucus and goblet cell hyperplasia, limited sub-epithelial oedema, squamous epithelial metaplasia, and lamina propria fibrosis were all identified.[247]

Figure 9.3: Example of CRSsNP tissue

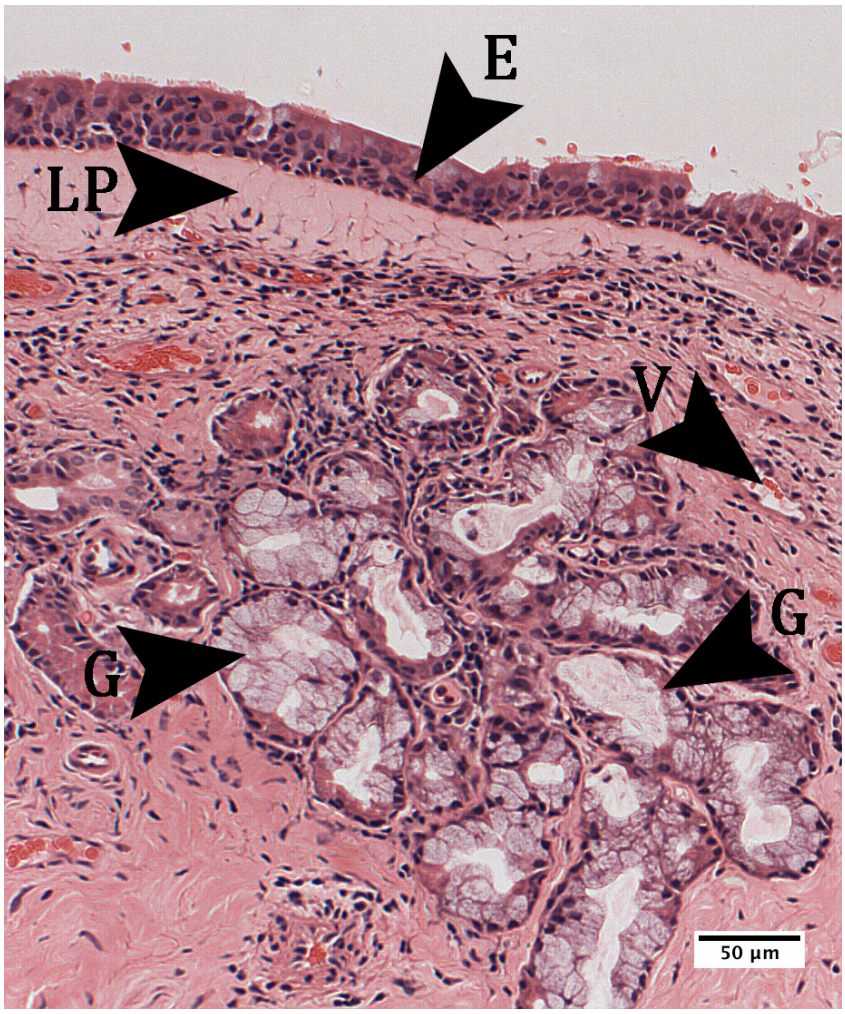

Figure 9.3: This photomicrograph is of one CRSsNP sinonasal tissue sample (haematoxalin and eosin [H&E] stain). Key structures were identified: (E) epithelium; (G) mucus cell hyperplasia in glands; (V) vasculature; and (LP) lamina propria fibrosis. An absence of sub-epithelial oedema was noted.

9. 2. 3 CRSwNP tissue

Key features of CRSwNP were noted in the CRSwNP tissue samples in this study, including sub-epithelial oedema, mucus and goblet cell hyperplasia, variable grades of epithelial cell thinning and destruction or squamous epithelial metaplasia, pseudocyst formation and atypical glandular formation (Figure 9.4, Figure 9.5). [222, 247, 257, 261]

Figure 9.4: Example of CRSwNP tissue (surface epithelium)

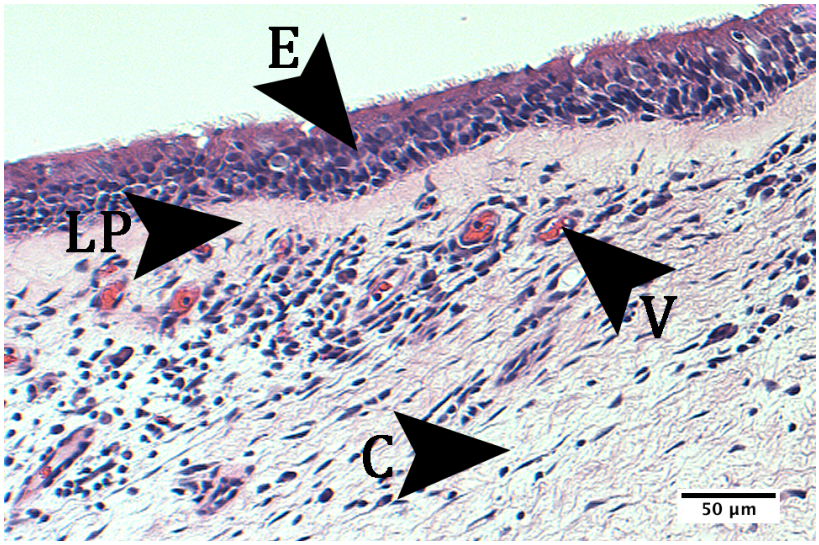

Figure 9.4 and Figure 9.5: These photomicrographs are of one CRSwNP sinonasal tissue sample (haematoxalin and eosin [H&E] stain). Key structures were identified: (E) epithelium; (G) mucus cell hyperplasia in glands and atypical glands; (V) vasculature; (LP) lamina propria; and (C) connective tissue including sub-epithelial oedema.

Figure 9.5: Example of CRSwNP tissue (glands)

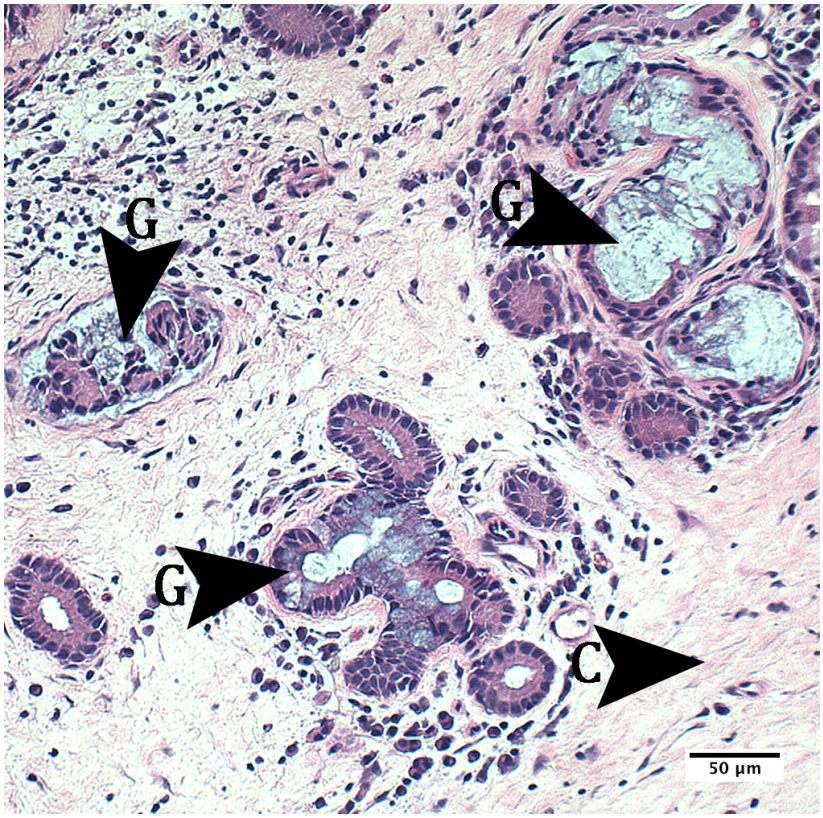

Figure 9.4 and Figure 9.5: These photomicrographs are of one CRSwNP sinonasal tissue sample (haematoxalin and eosin [H&E] stain). Key structures were identified: (E) epithelium; (G) mucus cell hyperplasia in glands and atypical glands; (V) vasculature; (LP) lamina propria; and (C) connective tissue including sub-epithelial oedema.

9. 3 AQP localisation in sinonasal tissue

9. 3. 1 Normal sinonasal tissue

Figure 9.6 demonstrates AQP localisation in one of the normal tissue samples from this study. In normal tissue, AQP1 was localised in vasculature ++ (range of scores from +/- to ++) and connective tissue + (- to ++) (Figure 9.7). AQP3 was localised strongly to the basolateral membrane of both surface +++ (- to +++) and glandular epithelium ++ (- to +++) and more weakly at the apical membrane and in the cell cytoplasm (Figure 9.8). AQP4 was localised weakly to the apical membrane of the surface epithelium +/- (- to +) and inconsistently in connective tissue + (+/- to +) (Figure 9.9). Strong localisation of AQP5 was observed at the apical ++ (- to +++) and basolateral membranes ++ (- to +++) of the surface epithelium, with less intense AQP5 localisation in the cytoplasm of these cells + (- to +) (Figure 9.10). AQP5 was also strongly localised to the apical membrane of the glandular epithelium +++ (- to +++), and with less intensity within the cell cytoplasm + (- to ++) and the basolateral membrane + (- to ++). Low levels of AQP7 were localised to the cytoplasm of the glandular epithelium - (- to ++), but this was inconsistent between specimens (Figure 9.11). Inconsistent and low levels of AQP11 were also observed in the cytoplasm of surface - (- to +/-) and glandular epithelia - (- to +/-) (Figure 9.12).

Figure 9.6: AQP localisation in normal sinonasal tissue samples

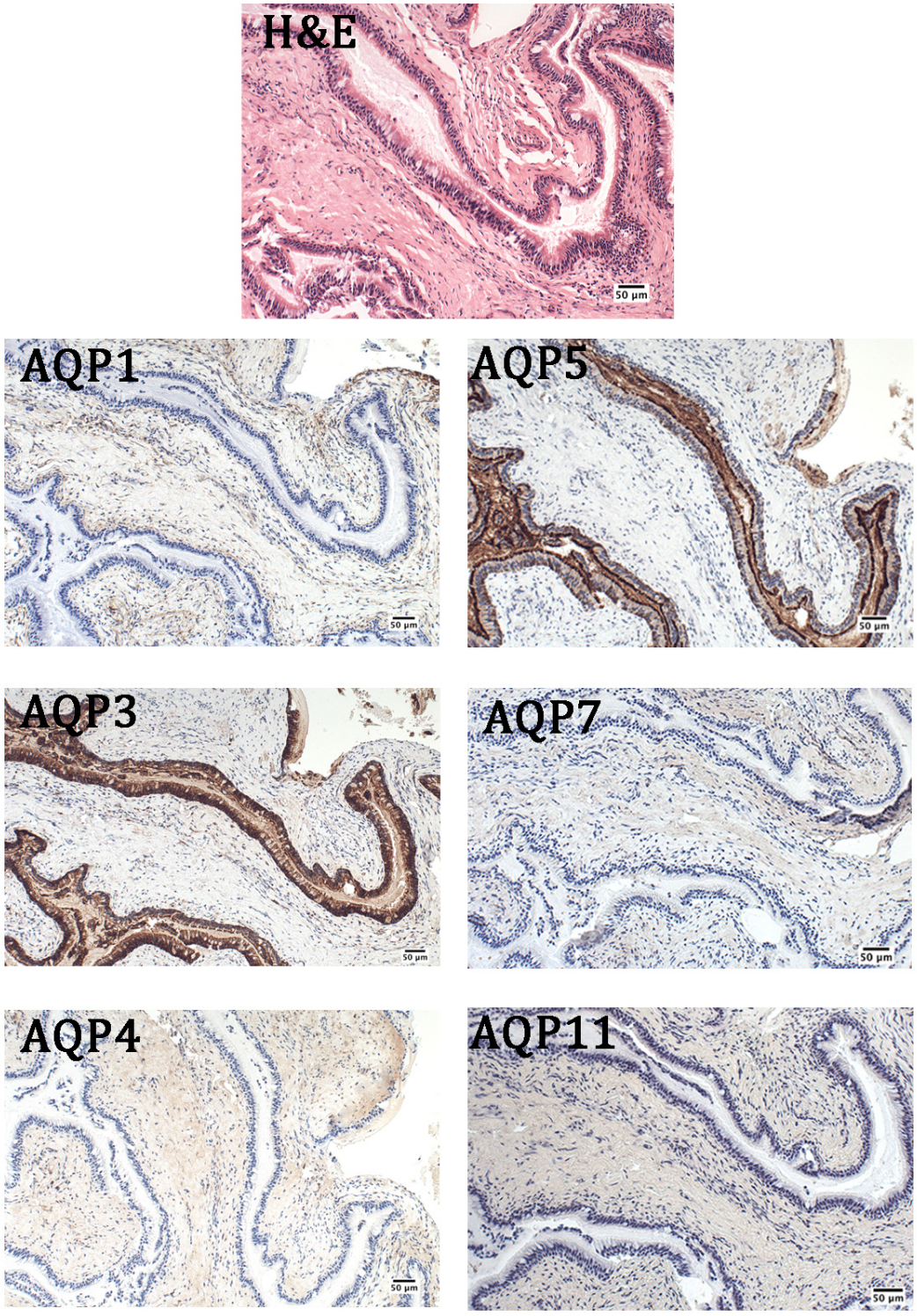

Figure 9.6: These photomicrographs demonstrate immunohistochemical (IHC) localisation of AQPs in normal sinonasal tissue (the same area of tissue in consecutive samples is photographed, with tissue stains labelled). The area of tissue photographed contains well-preserved surface epithelium, with minimal glandular epithelium.

Figure 9.7: Normal sinonasal tissue – AQP1

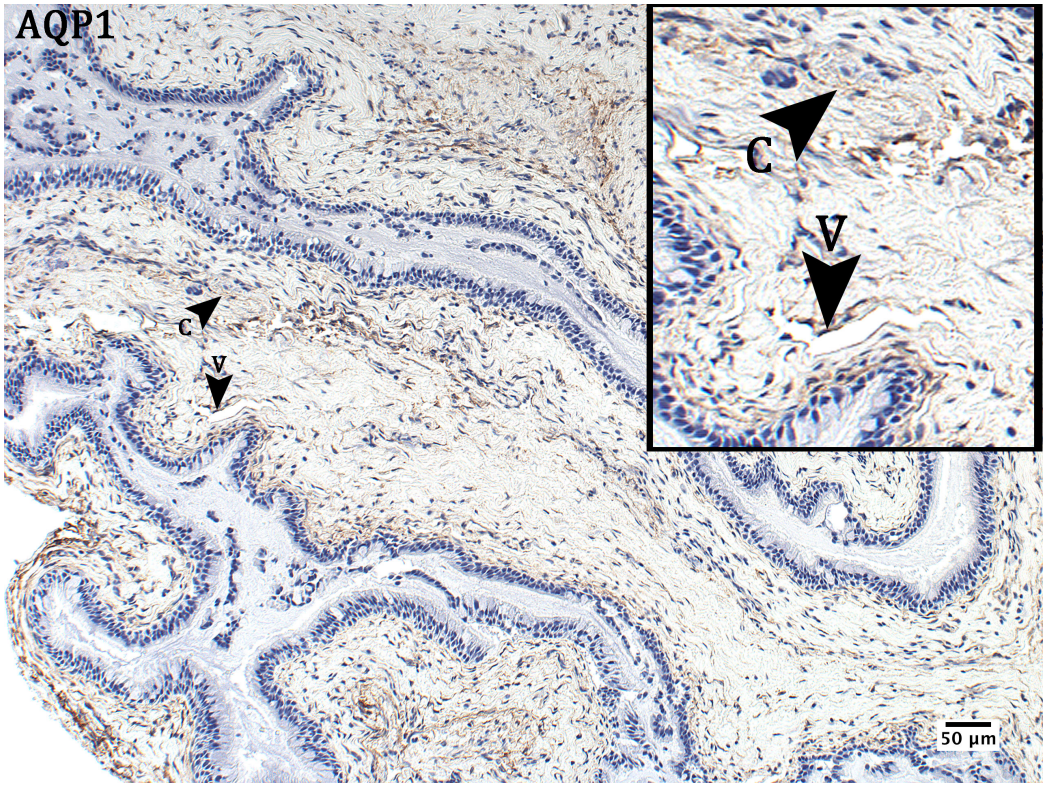

Figure 9.7: Photomicrograph of normal sinonasal tissue. AQP1 was localised to (C) connective tissue and (V) endothelium of blood vessels.

Figure 9.8: Normal sinonasal tissue – AQP3

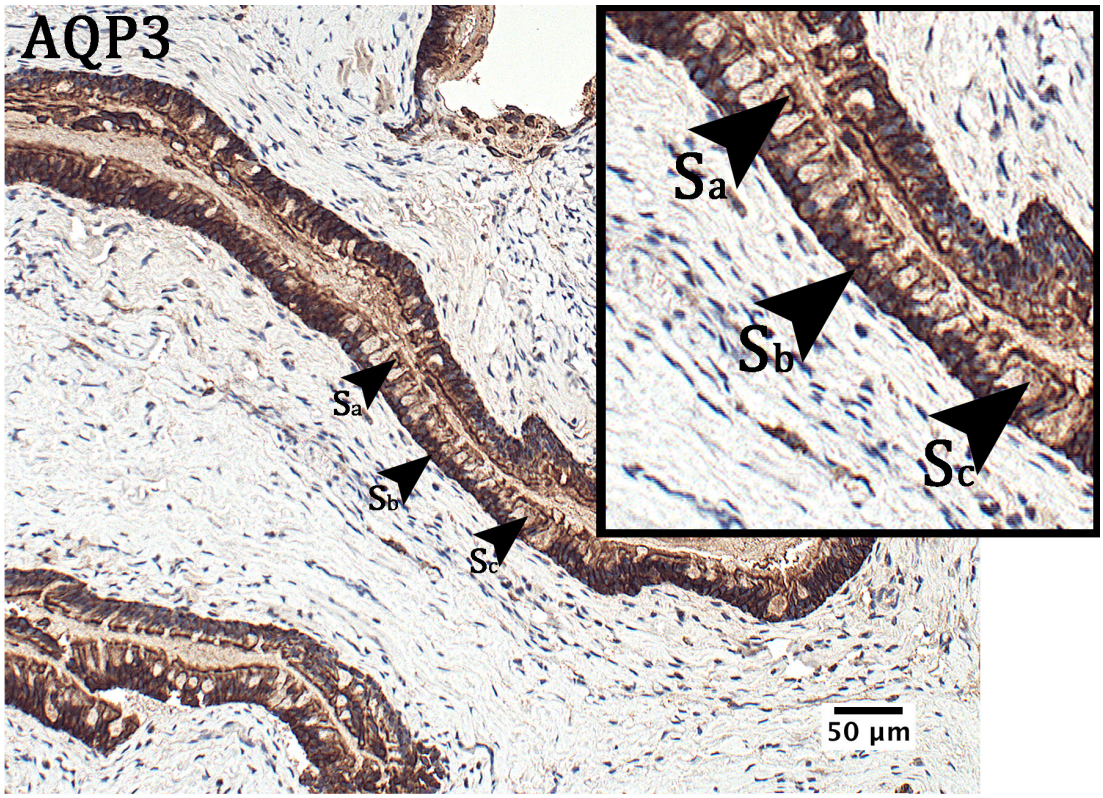

Figure 9.8: Photomicrograph of normal sinonasal tissue. AQP3 was localised to the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane; and (Sc) cytoplasm.

Figure 9.9: Normal sinonasal tissue – AQP4

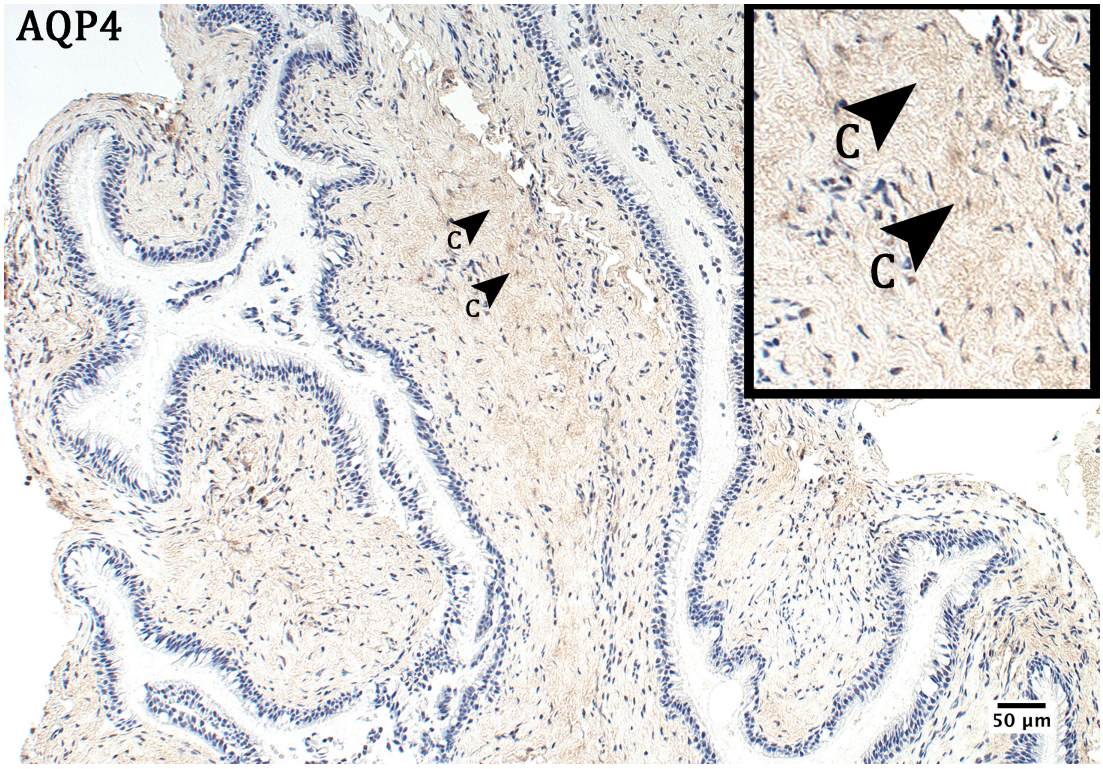

Figure 9.9: Photomicrograph of normal sinonasal tissue. AQP4 was localised to (C) connective tissue only.

Figure 9.10: Normal sinonasal tissue – AQP5

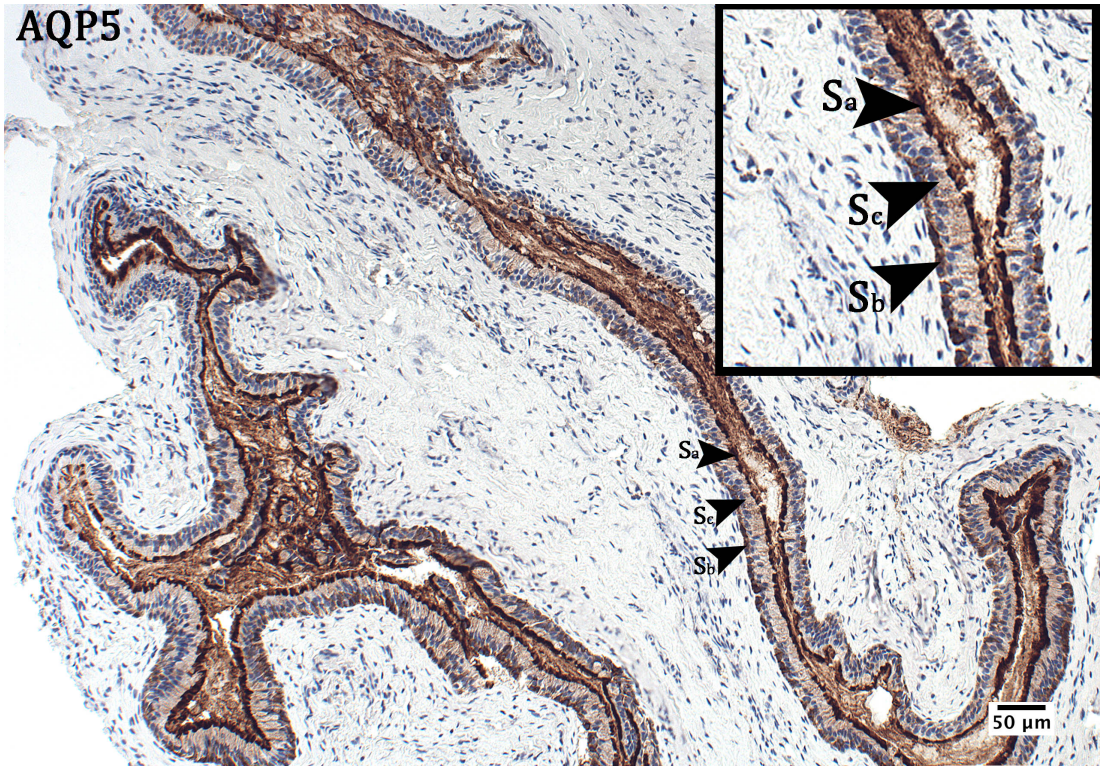

Figure 9.10: Photomicrograph of normal sinonasal tissue. AQP5 was localised to the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane; and (Sc) cytoplasm.

Figure 9.11: Normal sinonasal tissue – AQP7

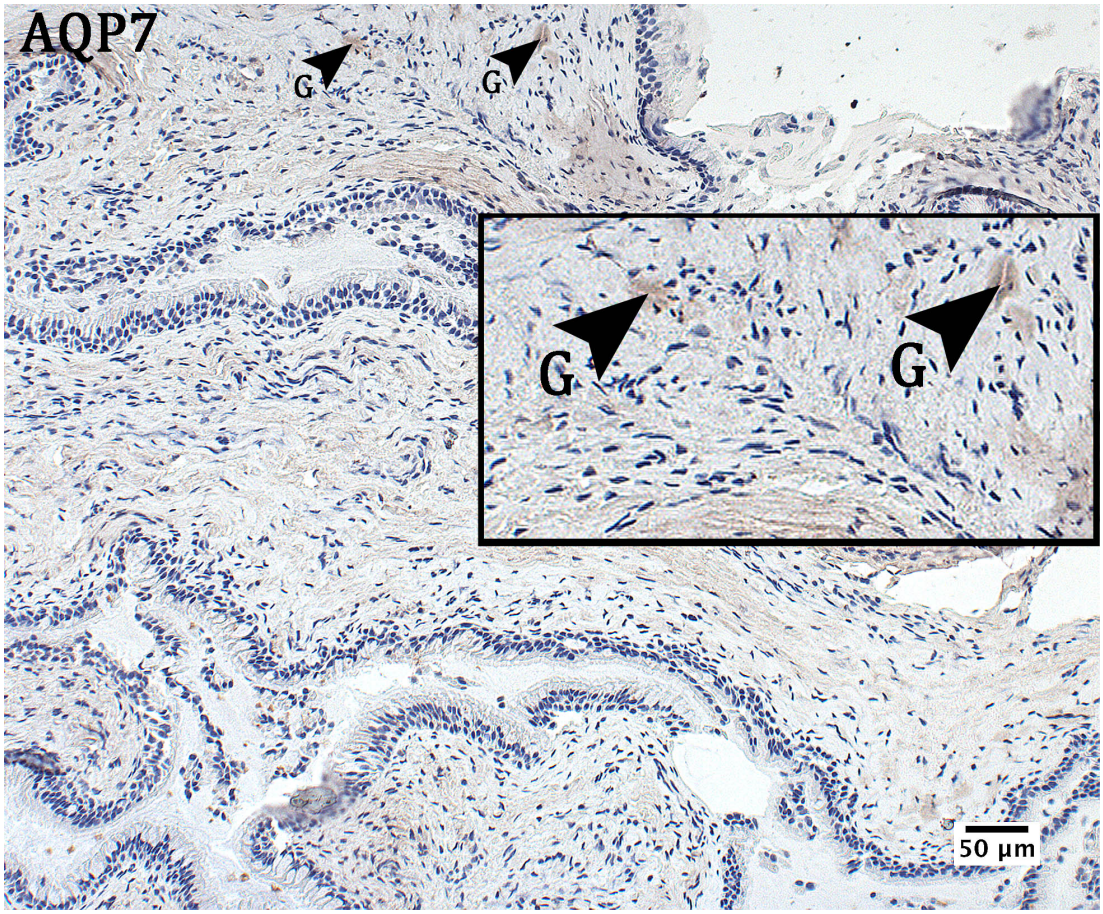

Figure 9.11: Photomicrograph of normal sinonasal tissue. AQP7 was localised to (G) cytoplasm of glandular epithelium.

Figure 9.12: Normal sinonasal tissue – AQP11

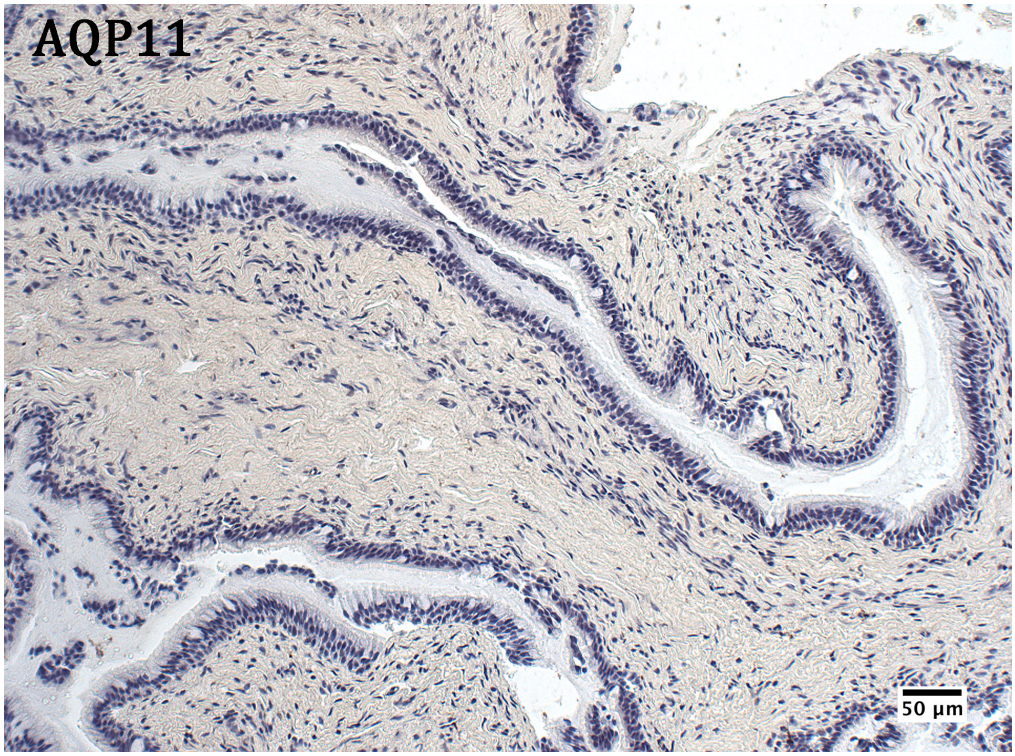

Figure 9.12: Photomicrograph of normal sinonasal tissue. AQP11 was inconsistently localised to cytoplasm of some surface and some glandular epithelium – not demonstrated in this sample.

9. 3. 2 CRSwNP sinonasal tissue

Figure 9.13 demonstrates AQP localisation in one of the CRSwNP tissue samples from this study. In the CRSwNP tissue, AQP1 was localised in vasculature ++ (range of scores from +/- to ++) and connective tissue ++ (- to +++) (Figure 9.14). AQP3 was localised to the basolateral membrane of both surface ++ (- to +++) and glandular epithelium ++ (- to +++) and more weakly at the apical membrane and in the cell cytoplasm (Figure 9.15). AQP4 was localised weakly to the cytoplasm of glandular epithelial cells + (- to ++) (Figure 9.16). Very strong localisation of AQP5 was observed at the apical surface of glandular epithelium +++ (all samples) (Figure 9.17). AQP5 was also localised to the basolateral surface of both surface epithelium ++ (++ to +++) and glandular epithelium ++ (++ to +++), and the cytoplasm of glandular epithelium ++ (++ to +++). Inconsistent and low levels of AQP7 were observed in the cytoplasm of surface + (+/- to +) and glandular epithelia +/- (- to +) (Figure 9.18). A low level of AQP11 was observed in the connective tissue only +/- (- to +/-); however, this was inconsistent between specimens (Figure 9.19).

Figure 9.13: AQP localisation in CRSwNP sinonasal tissue samples

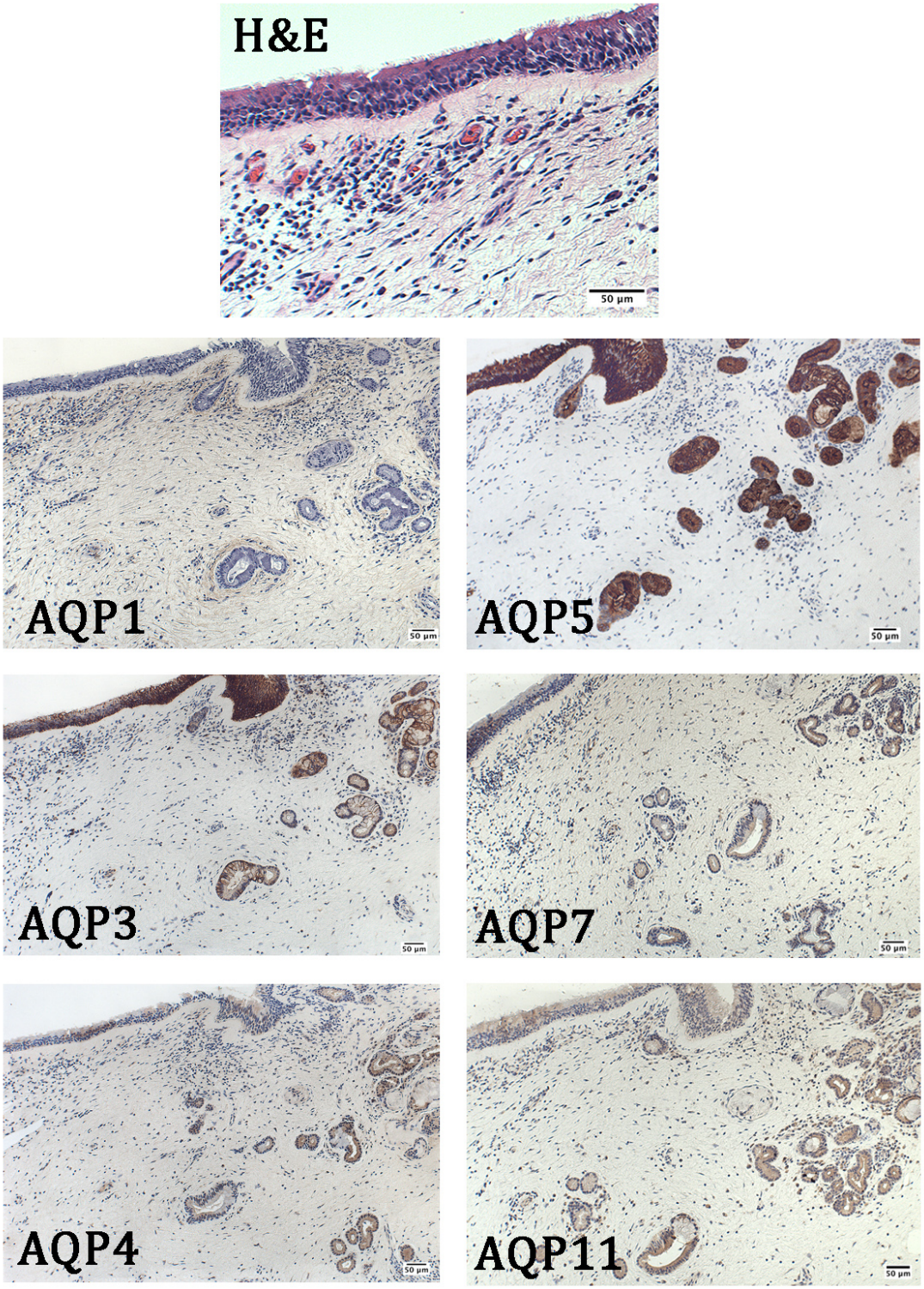

Figure 9.13: These photomicrographs demonstrate immunohistochemical (IHC) localisation of AQPs in sinonasal tissue from a CRSwNP patient (approximately the same area of consecutive tissue sample slices was photographed). The area of tissue photographed contains surface and glandular epithelium, and fibrotic connective tissue.

Figure 9.14: CRSwNP sinonasal tissue – AQP1

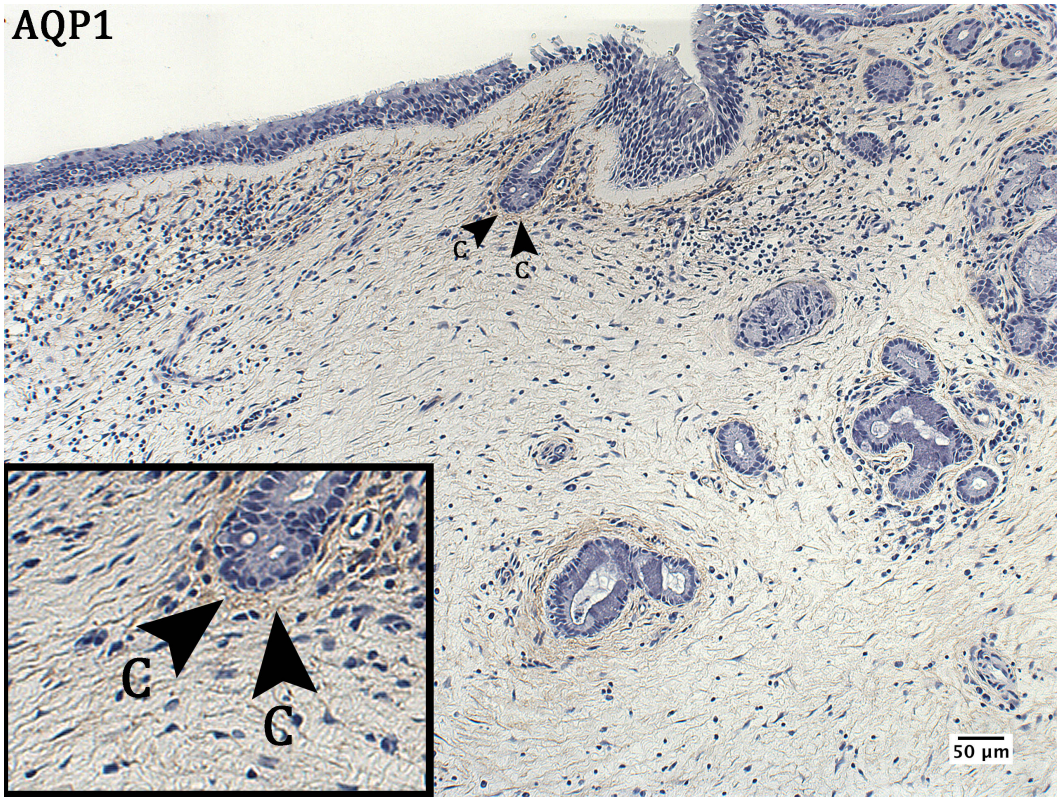

Figure 9.14: Photomicrograph of CRSwNP sinonasal tissue. AQP1 was localised to (C) connective tissue and endothelium of blood vessels (not seen here).

Figure 9.15: CRSwNP sinonasal tissue – AQP3

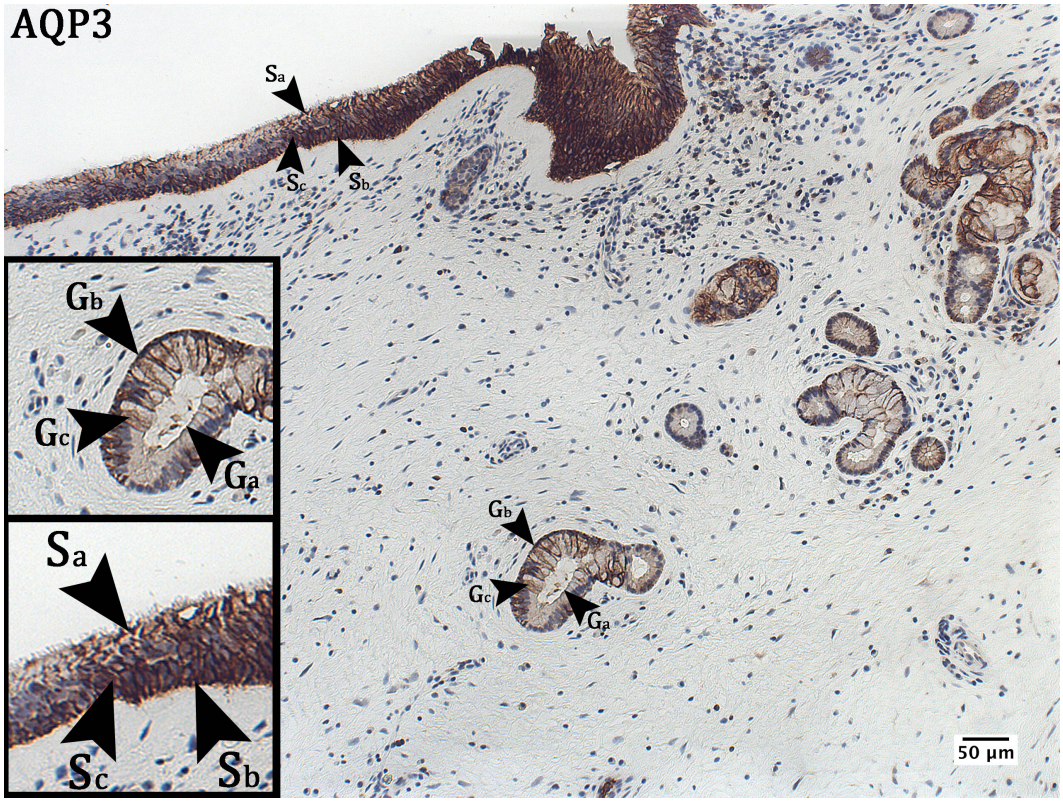

Figure 9.15: Photomicrograph of CRSwNP sinonasal tissue. AQP3 was localised to the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane; and (Sc) cytoplasm. It was also localised to the glandular epithelium: (Ga) apical membrane; (Gb) basolateral membrane; and (Gc) cytoplasm.

Figure 9.16: CRSwNP sinonasal tissue – AQP4

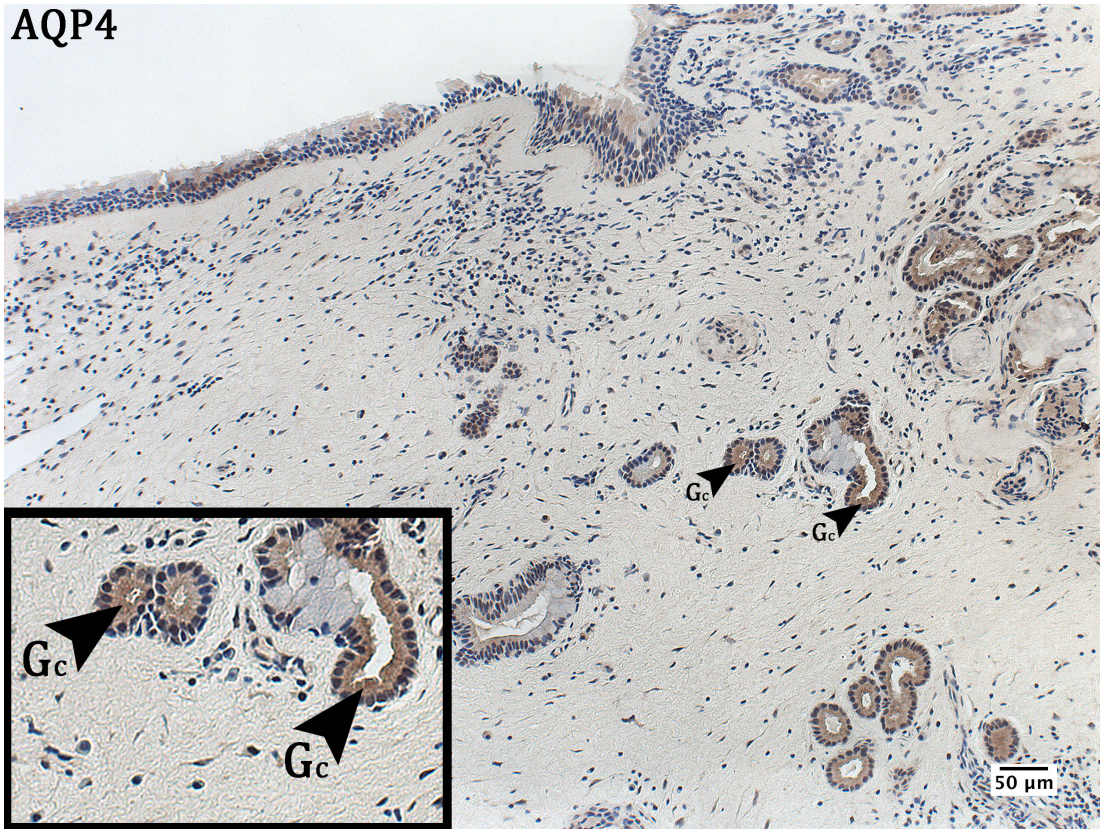

Figure 9.16: Photomicrograph of CRSwNP sinonasal tissue. AQP4 was localised to the (Gc) cytoplasm of glandular epithelium.

Figure 9.17: CRSwNP sinonasal tissue – AQP5

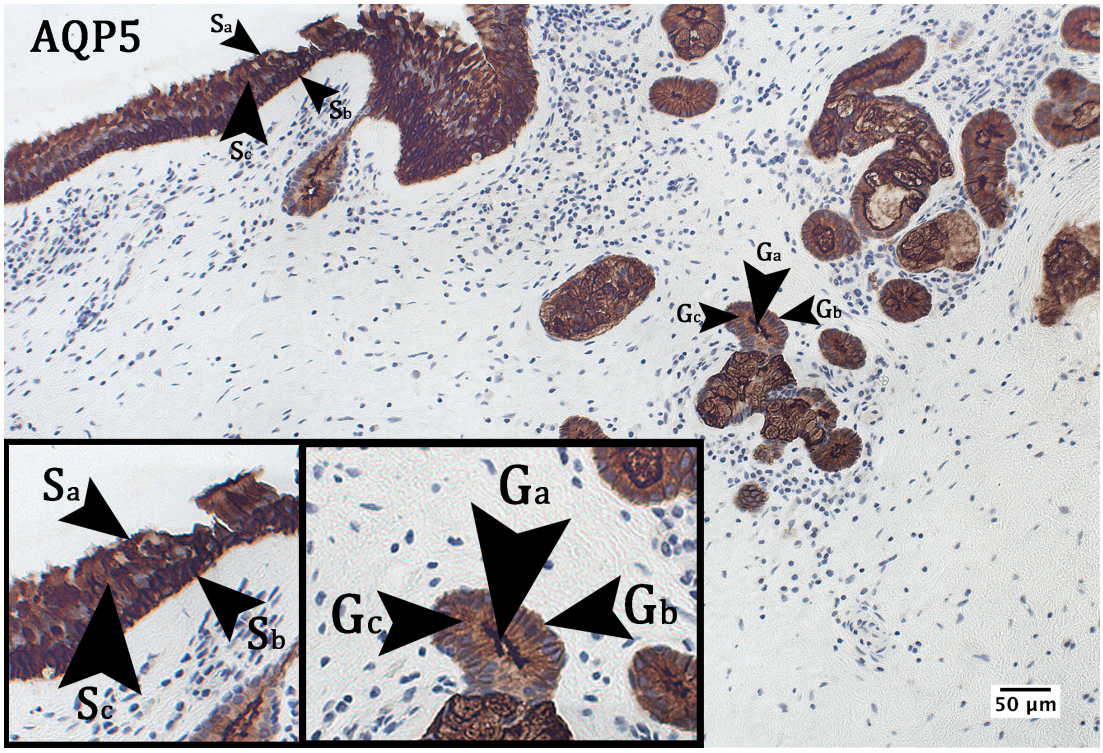

Figure 9.17: Photomicrograph of CRSwNP sinonasal tissue. AQP5 was localised to the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane; and (Sc) cytoplasm. AQP5 was also localised to the glandular epithelium: (Ga) apical membrane; (Gb) basolateral membrane; and (Gc) cytoplasm.

Figure 9.18: CRSwNP sinonasal tissue – AQP7

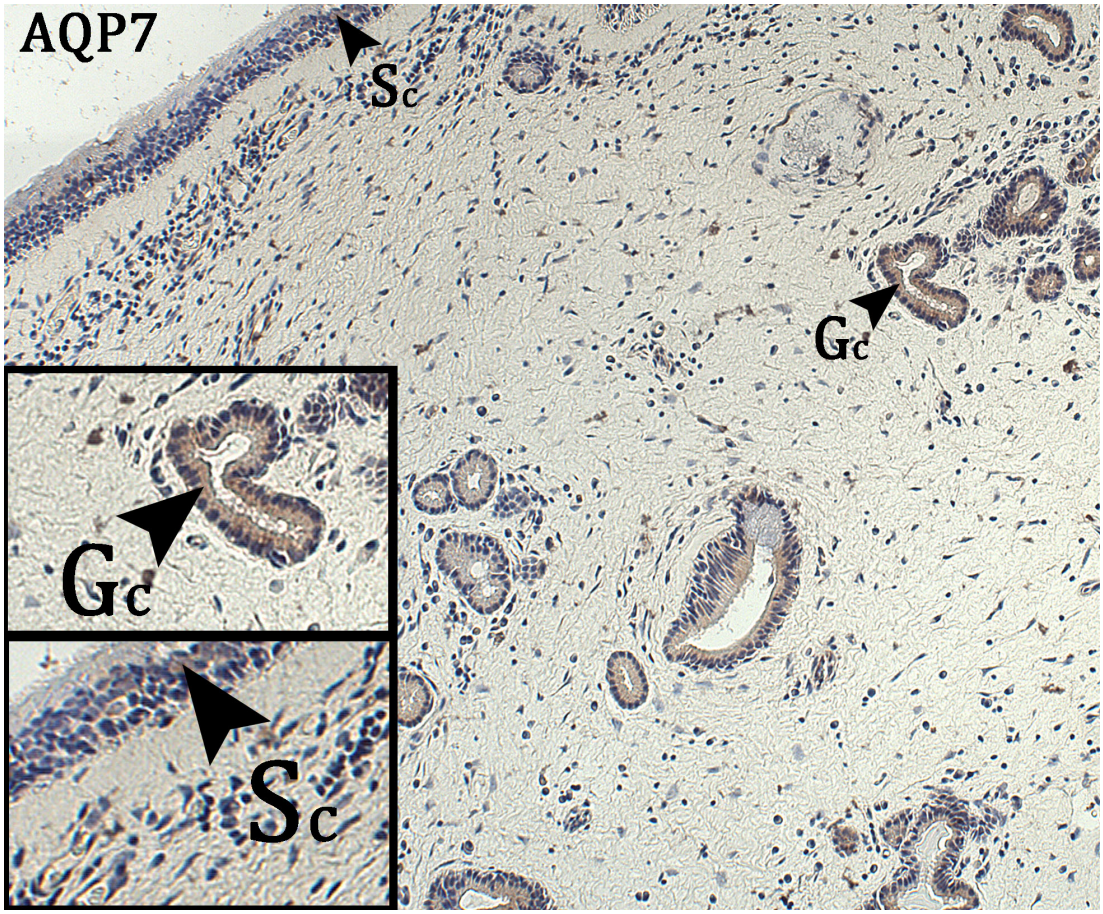

Figure 9.18: Photomicrograph of CRSwNP sinonasal tissue. AQP7 was localised to cytoplasm of (Sc) surface epithelium and (Gc) glandular epithelium.

Figure 9.19: CRSwNP sinonasal tissue – AQP11

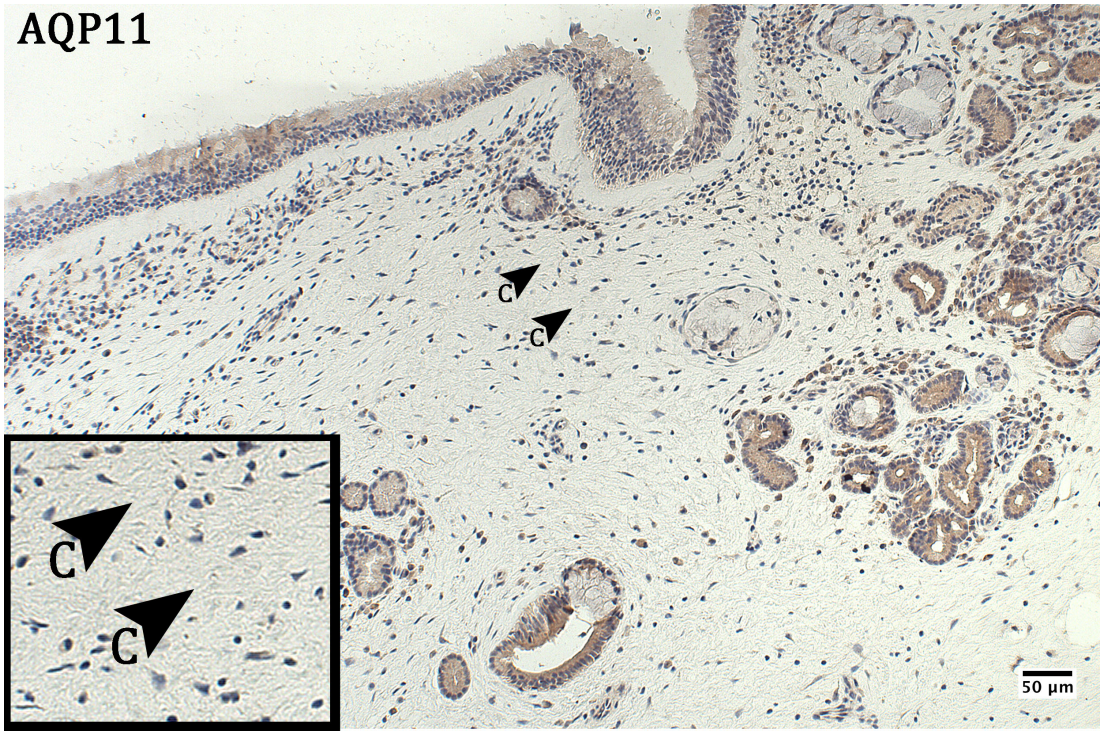

Figure 9.19: Photomicrograph of CRSwNP sinonasal tissue. AQP11 was inconsistently localised to (C) connective tissue of some samples. This tissue sample was the only one to demonstrate AQP11 localisation to glandular epithelial cytoplasm.

9. 3. 3 CRSsNP sinonasal tissue

Figure 9.20 demonstrates AQP localisation in one of the CRSsNP tissue samples from this study. In CRSsNP tissue, AQP1 was localised in vasculature ++ (range of scores from + to ++) and connective tissue ++ (+ to +++) (Figure 9.21). AQP3 was strongly localised to the basolateral membrane of both surface +++ (- to +++) and glandular epithelium +++ (- to +++) and less strongly at the apical surface of surface epithelium + (- to ++) and within the cytoplasm of surface + (- to +) and glandular epithelia + (+/- to +) (Figure 9.22). AQP4 was localised to the cytoplasm of glandular epithelial cells ++ (- to ++) and throughout the surface epithelial cells: apex and basolateral membranes and cytoplasm were all + (- to ++) (Figure 9.23). AQP4 was also localised to the connective tissue + (- to ++), and at the apices of some glandular epithelial cells - (- to +++). A very strong expression of AQP5 was observed at the apical surface of glandular +++ (all samples) and surface epithelia +++ (all samples) as well as at the basolateral membrane of the surface epithelium +++ (all samples) (Figure 9.24). Strong localisation of AQP5 was seen in the cytoplasm of the surface epithelium ++ (all samples) and at the basolateral surface of glandular epithelium ++ (++ to +++). AQP5 was also localised within the cytoplasm of glandular epithelium + (- to ++). Inconsistent and low levels of AQP7 were observed in the cytoplasm of surface + (- to +) and glandular epithelia +/- (- to +) (Figure 9.25). A low level of AQP11 was observed in the connective tissue +/- (- to +/-) and the cytoplasm of both surface - (- to +/-) and glandular epithelia - (- to +/-); however, this was inconsistent between specimens (Figure 9.26).

Figure 9.20: AQP localisation in CRSsNP sinonasal tissue samples

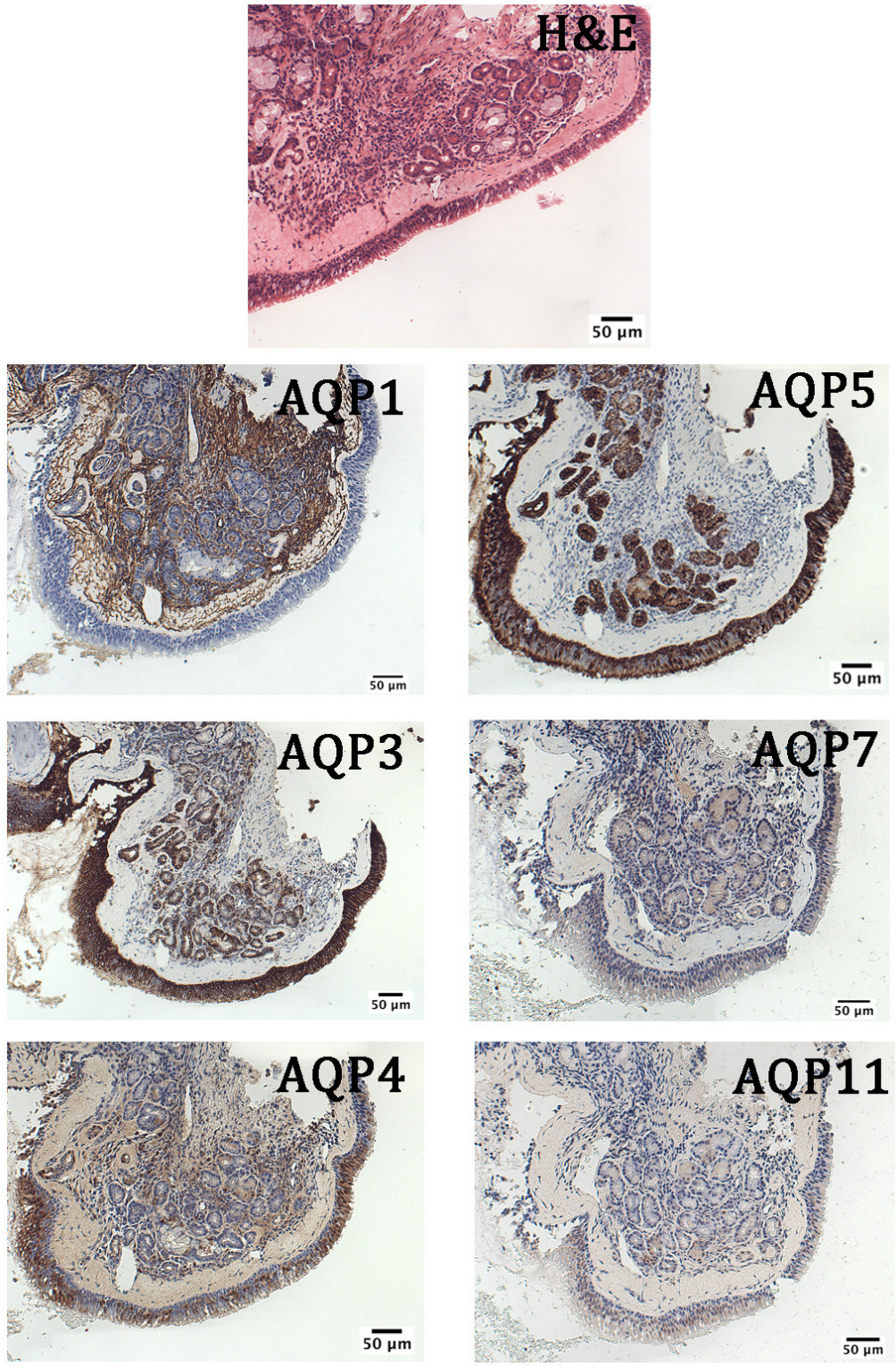

Figure 9.20: These photomicrographs demonstrate immunohistochemical (IHC) localisation of AQPs in sinonasal tissue from a CRSsNP patient (approximately the same area of consecutive tissue sample slices was photographed). The area of tissue photographed contains both surface and glandular epithelium.

Figure 9.21: CRSsNP sinonasal tissue – AQP1

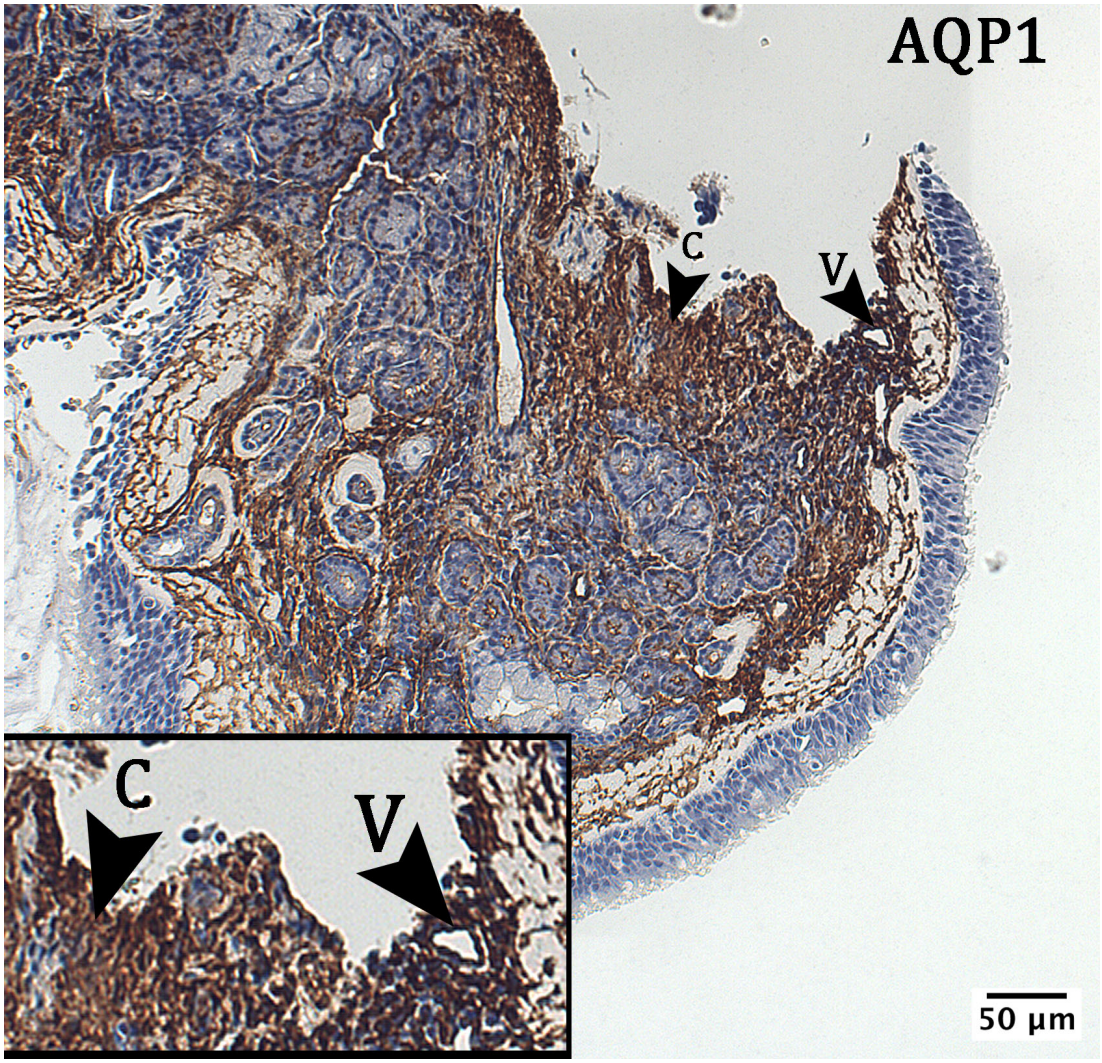

Figure 9.21: Photomicrograph of CRSsNP sinonasal tissue. AQP1 was localised to (C) connective tissue and (V) endothelium of blood vessels.

Figure 9.22: CRSsNP sinonasal tissue – AQP3

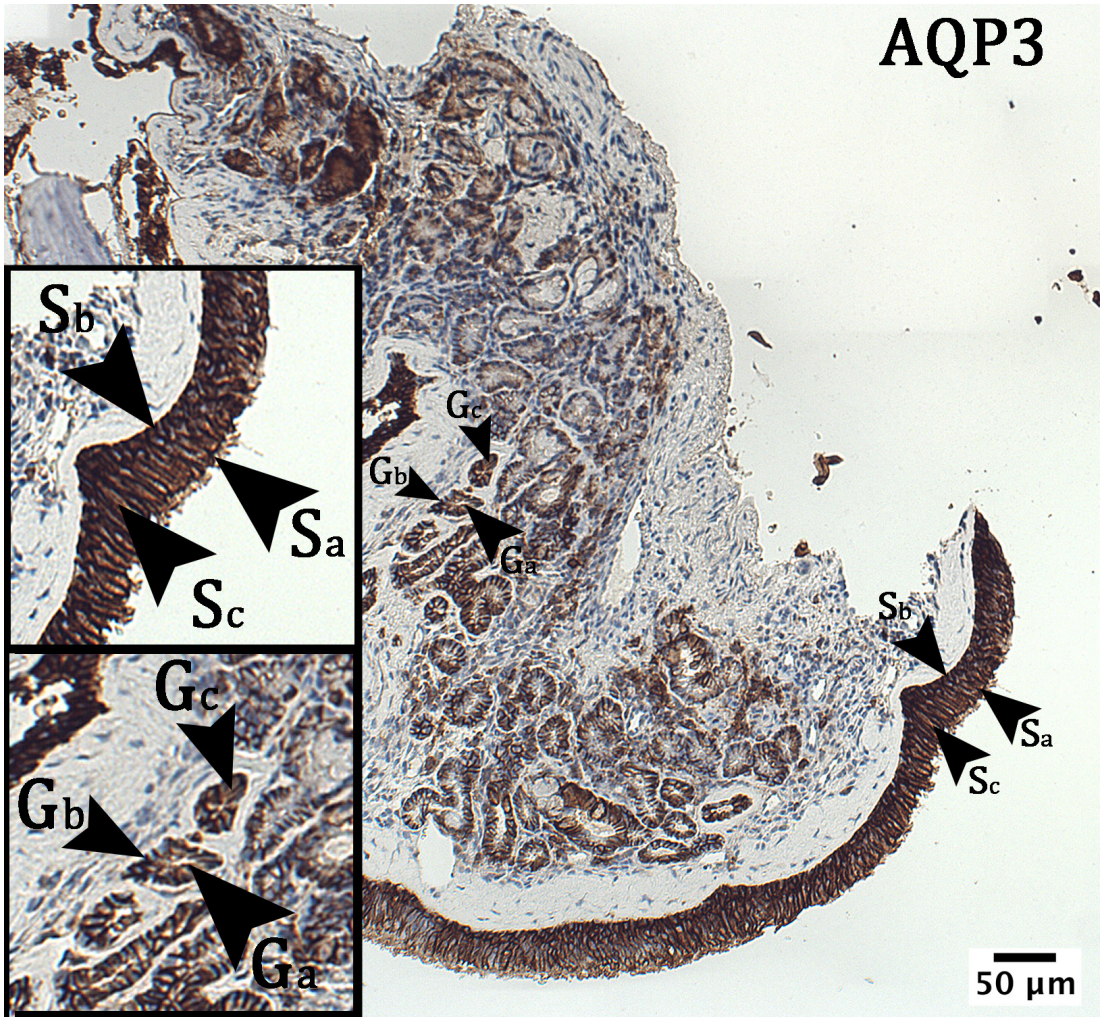

Figure 9.22: Photomicrograph of CRSsNP sinonasal tissue. AQP3 was localised to the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane; and (Sc) cytoplasm. AQP3 was also localised to the glandular epithelium: (Gb) basolateral membrane and (Gc) cytoplasm.

Figure 9.23: CRSsNP sinonasal tissue – AQP4

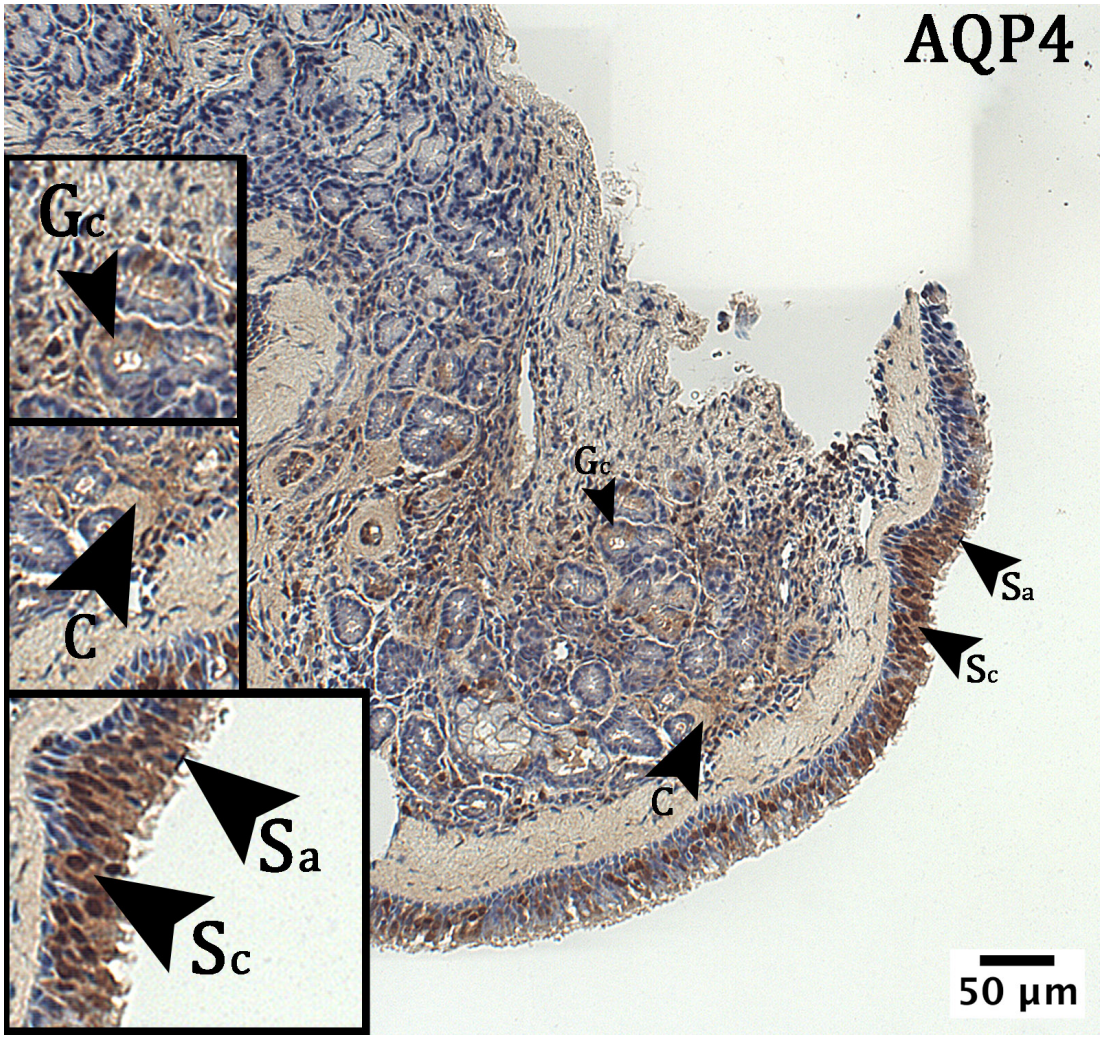

Figure 9.23: Photomicrograph of CRSsNP sinonasal tissue. AQP4 was localised to the glandular epithelium: (Gc) cytoplasm and (Ga) apical membrane (latter not seen here) and the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane (not clearly demonstrated in this tissue sample); (Sc) cytoplasm; and (C) connective tissue.

Figure 9.24: CRSsNP sinonasal tissue – AQP5

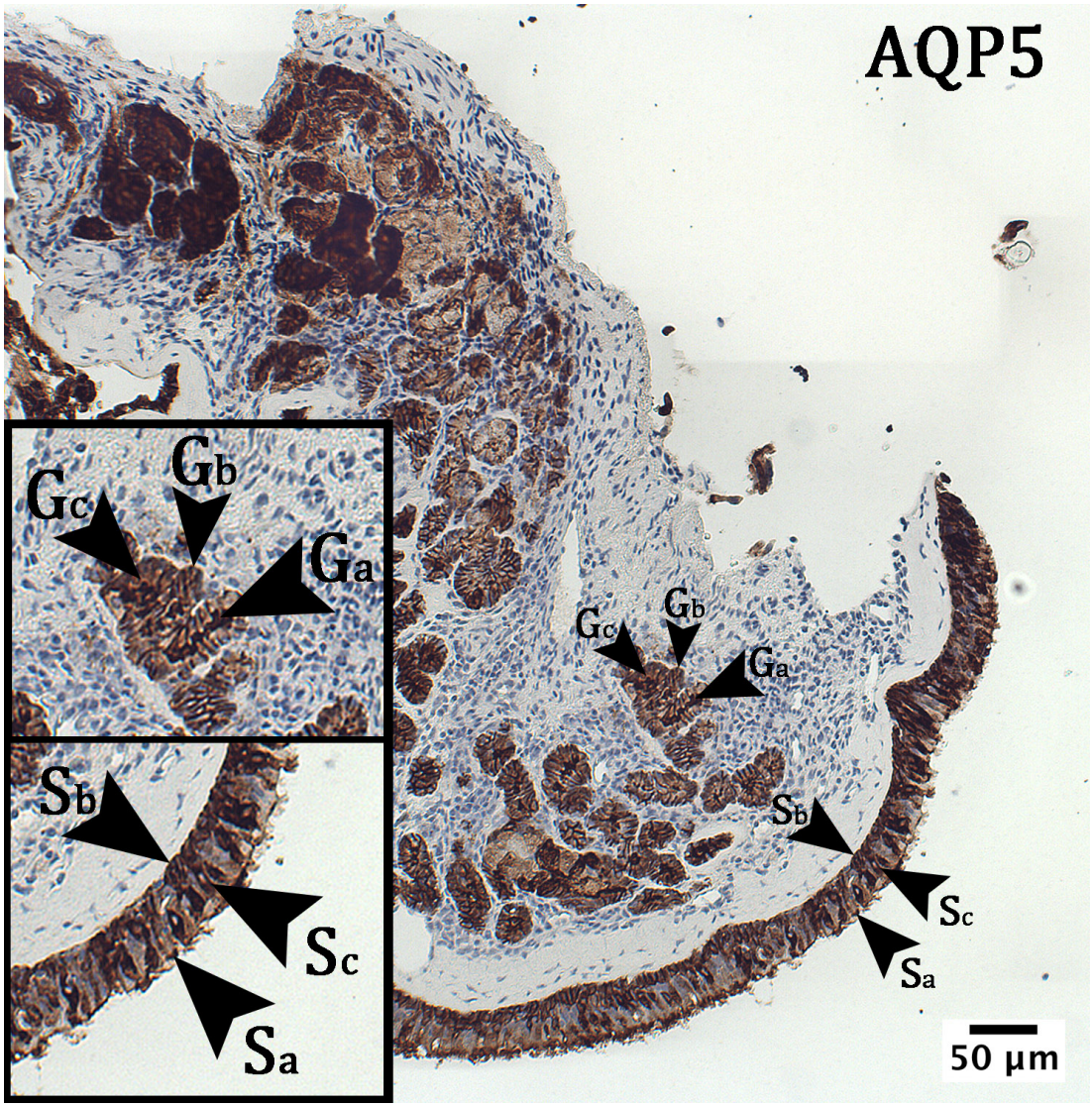

Figure 9.24: Photomicrograph of CRSsNP sinonasal tissue. AQP5 was localised to the glandular epithelium: (Ga) apical membrane; (Gb) basolateral membrane; and (Gc) cytoplasm and the surface epithelium: (Sa) apical membrane; (Sb) basolateral membrane; and (Sc) cytoplasm.

Figure 9.25: CRSsNP sinonasal tissue – AQP7

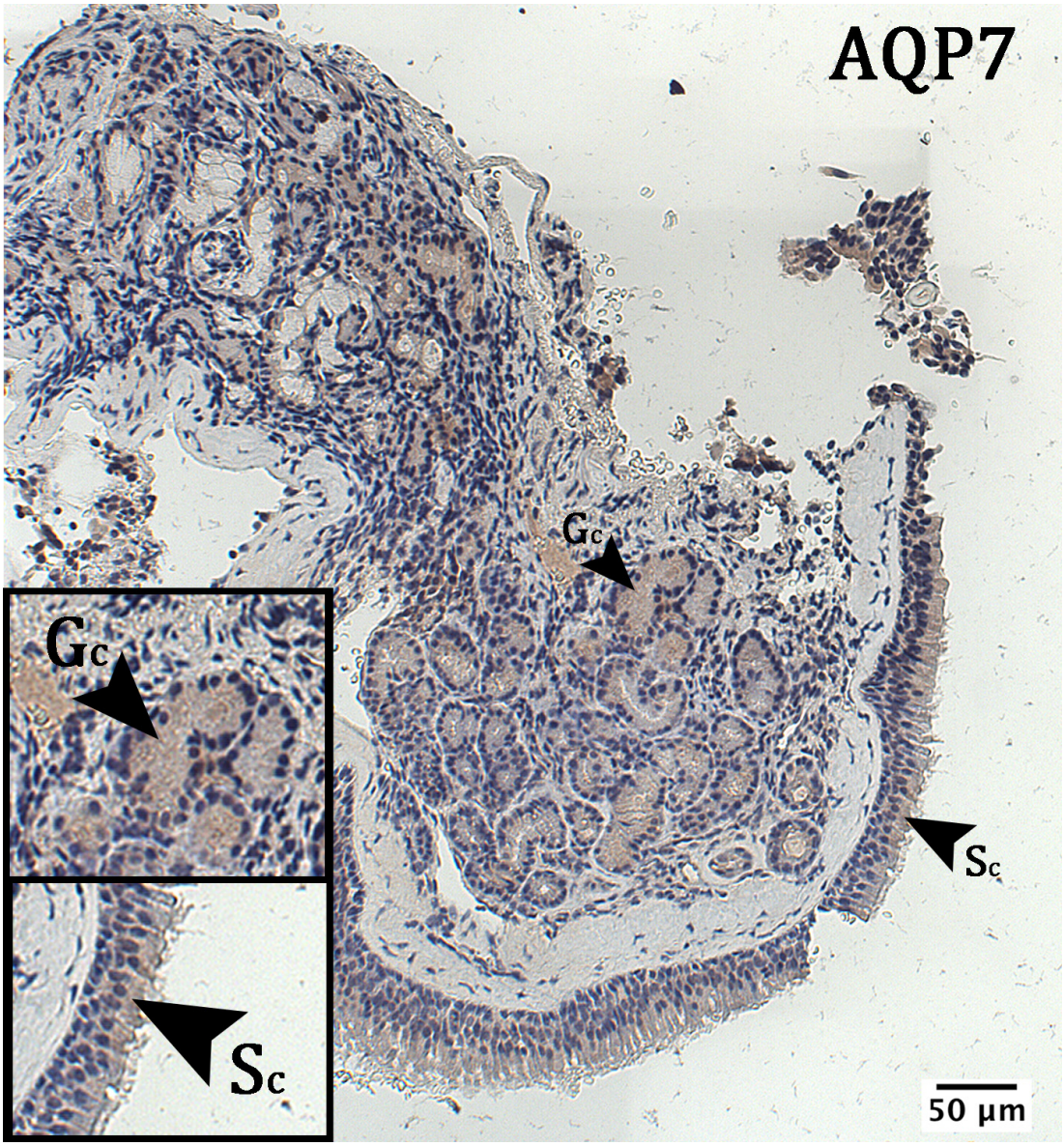

Figure 9.25: Photomicrograph of CRSsNP sinonasal tissue. AQP7 was localised to (Gc) cytoplasm of glandular epithelium and inconsistently localised to cytoplasm of some (Sc) surface epithlium.

Figure 9.26: CRSsNP sinonasal tissue – AQP11

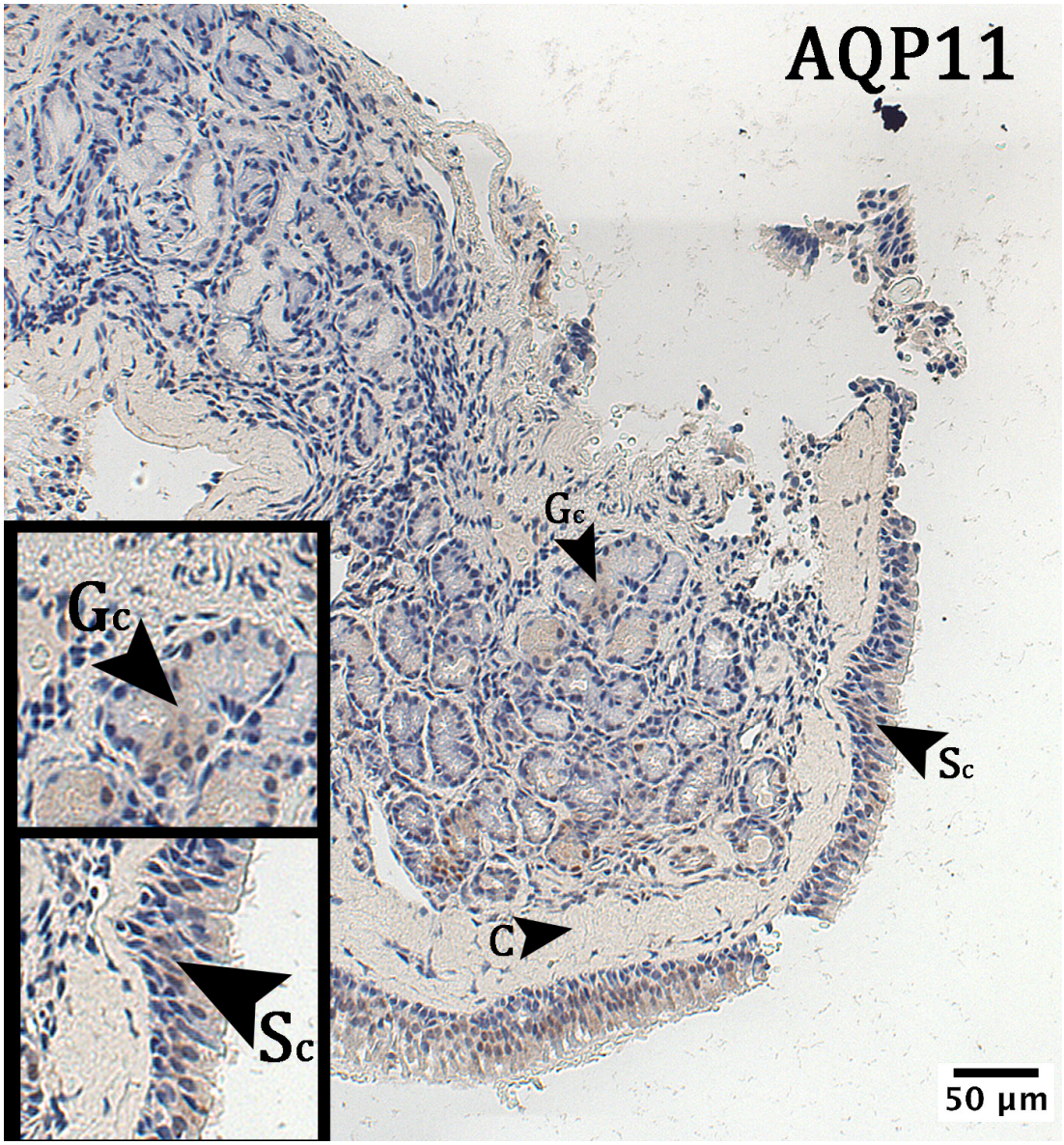

Figure 9.26: Photomicrograph of CRSsNP sinonasal tissue. AQP11 was inconsistently localised to cytoplasm of some (Sc) surface and some (Gc) glandular epithelium, and to (C) connective tissue (poor labelling seen in this tissue).

9. 4 Comparison of AQP localisation: Normal vs. CRSwNP vs. CRSsNP

A localisation intensity score was given for each AQP in every tissue sample at every sub-site using the semi-quantitative Likert scale. A median score for each group was calculated and tabulated (including the range of scores at the sub-site) (Table 9.2). Sub-group analyses were performed to compare normal controls to CRSwNP and controls to CRSsNP. Statistically significant differences in the localisation intensity of AQPs were identified at three sub-sites (Table 9.3). AQP4 was localised more intensely in the cytoplasm of glandular epithelial cells in CRSsNP (++ compared to +, $p<0.05$) (Table 9.3, Figure 9.27). AQP5 was labelled more intensely in the cytoplasm of both the surface epithelium in CRSsNP (++ to +, $p<0.05$) (Figure 9.28), and in glandular epithelial cells in CRSwNP (++ to +, $p<0.05$) (Figure 9.29) when compared to the control tissue.

Table 9.2: Immunohistochemistry localisation intensity scores by location

AQP	Disease Group	Surface Epithelium Apical	Surface Epithelium Basolateral	Surface Epithelium Cytoplasm	Gland Epithelium Apical	Gland Epithelium Basolateral	Gland Epithelium Cytoplasm	Vasculature	Connective Tissue
AQP1	Normal	-	-	-	-	-	-	++ (+/- to ++)	+ (- to ++)
	CRSwNP	-	-	-	-	-	-	++ (+/- to ++)	++ (+ to +++)
	CRSsNP	-	-	-	-	-	-	++ (+ to ++)	++ (+ to +++)
AQP3	Normal	+ (- to ++)	+++ (- to +++)	++ (- to +)	+/- (- to +)	++ (- to +++)	- (- to ++)	-	-
	CRSwNP	+ (- to ++)	++ (- to +++)	+ (- to ++)	+/- (- to +)	++ (+ to +++)	+ (+/- to +)	-	-
	CRSsNP	+ (- to ++)	+++ (- to +++)	+ (- to ++)	+/- (- to +)	+++ (+ to +++)	+ (+/- to +)	-	- (- to +/-)
AQP4	Normal	+/- (- to +)	- (- to +)	+/- (- to +)	- (- to +/-)	- (- to +/-)	- (- to ++)	-	+ (+/- to +)
	CRSwNP	- (- to +)	-	- (- to ++)	- (- to +/-)	- (- to +/-)	+ (- to ++)	-	+ (+/- to ++)
	CRSsNP	+ (- to ++)	+ (- to ++)	+ (- to ++)	- (- to +++)	+/- (- to ++)	**++ (- to ++)**	-	+ (- to ++)
AQP5	Normal	++ (- to +++)	++ (- to +++)	+ (- to +)	+++ (- to +++)	+ (- to +)	+ (- to ++)	-	-
	CRSwNP	+ (+ to +++)	++ (++ to +++)	**+ (+ to ++)**	+++	++ (+ to ++)	++ (+ to ++)	-	-
	CRSsNP	+++	+++	++	+++	++ (++ to +++)	**+ (+ to ++)**	-	-
AQP7	Normal	-	-	-	-	-	- (- to ++)	-	-
	CRSwNP	-	-	+ (+/- to +)	-	-	+/- (- to +)	-	-
	CRSsNP	-	-	+ (- to +)	-	-	+/- (- to +)	-	-
AQP11	Normal	-	-	- (- to +/-)	-	-	- (- to +/-)	-	- (- to +/-)
	CRSwNP	-	-	-	-	-	-	-	+/- (- to +/-)
	CRSsNP	-	-	- (- to +/-)	-	-	- (- to +/-)	-	+/- (- to +/-)

Table 9.2: Immunohistochemistry (IHC) localisation intensity scores are presented by location. All tissue sections were scored for the intensity of immunohistochemical (IHC) AQP labelling at the anatomical locations listed in the table using a Likert scale: -, +/-, +, ++ and +++ or "not present" when the anatomical structure was not present in the section. Data were presented as a median (range). The absence of range indicates that all scores were the same. Boxes shaded grey indicate that localisation was significantly different to the control ($p<0.05$ Kruskal–Wallis with Dunn's correction for multiple comparisons).

Table 9.3: Altered localisation of AQP0–AQP11 in sinonasal tissue in CRS

AQP	Anatomical region	Intra-cellular subsite	Kruskal-Wallis p value	Control vs. CRSwNP Dunns	Control vs. CRSsNP Dunns
AQP1	Surface epithelium	Apical	No difference		
		Basolateral	No difference		
		Cytoplasm	No difference		
	Glandular epithelium	Apical	No difference		
		Basolateral	No difference		
		Cytoplasm	No difference		
	Vasculature		No difference		
	Connective tissue		0.066		
AQP3	Surface epithelium	Apical	No difference		
		Basolateral	0.720		
		Cytoplasm	No difference		
	Glandular epithelium	Apical	No difference		
		Basolateral	0.105		
		Cytoplasm	0.056		
	Vasculature		No difference		
	Connective tissue		No difference		
AQP4	Surface epithelium	Apical	0.287		
		Basolateral	**0.047**	ns	ns
		Cytoplasm	0.337		
	Glandular epithelium	Apical	No difference		
		Basolateral	**0.048**	ns	ns
		Cytoplasm	**0.039**	ns	Yes
	Vasculature		No difference		
	Connective tissue		No difference		
AQP5	Surface epithelium	Apical	0.814		
		Basolateral	0.199		
		Cytoplasm	**0.025**	ns	Yes
	Glandular epithelium	Apical	No difference		
		Basolateral	0.055		
		Cytoplasm	**0.041**	Yes	ns
	Vasculature		No difference		
	Connective tissue		No difference		
AQP7	Surface epithelium	Apical	No difference		
		Basolateral	No difference		
		Cytoplasm	0.078		
	Glandular epithelium	Apical	No difference		
		Basolateral	No difference		
		Cytoplasm	0.357		
	Vasculature		No difference		
	Connective tissue		No difference		
AQP11	Surface epithelium	Apical	No difference		
		Basolateral	No difference		
		Cytoplasm	No difference		
	Glandular epithelium	Apical	No difference		
		Basolateral	No difference		
		Cytoplasm	No difference		
	Vasculature		No difference		
	Connective tissue		0.432		

Table 9.3: The table lists all anatomical sub-sites where AQP localisation intensity was scored, and the comparisons between normal vs. CRSwNP vs. CRSsNP using Kruskal-Wallis with Dunn's correction for multiple comparisons. Significant differences were identified between normal and CRSsNP tissue in AQP4 localisation

intensity in the cytoplasm of glandular epithelium, and in AQP5 in the cytoplasm of surface epithelium. A significant difference was also found between normal and CRSwNP tissue in AQP5 in the cytoplasm of glandular epithelium.

Figure 9.27: AQP4 intensity in glandular epithelium – Normal vs. CRSsNP

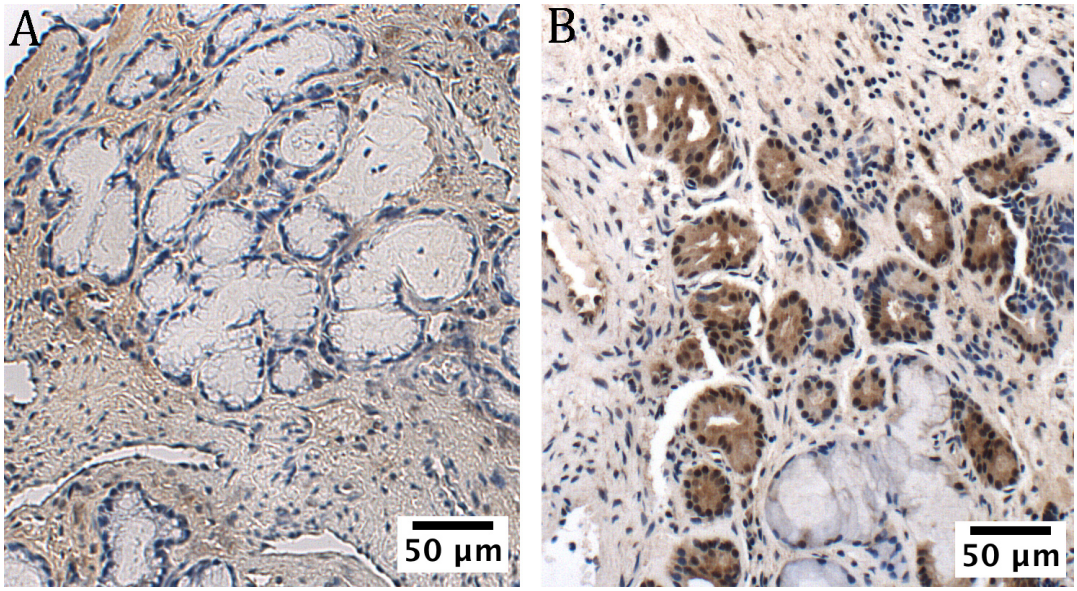

Figure 9.27: These photomicrographs demonstrate AQP4 localisation in glandular epithelium in (A) normal sinonasal tissue and (B) CRSsNP sinonasal tissue. More intense localisation of AQP4 is seen in the cytoplasm of glandular epithelial cells in CRSsNP tissue.

Figure 9.28: AQP5 intensity in surface epithelium – Normal vs. CRSsNP

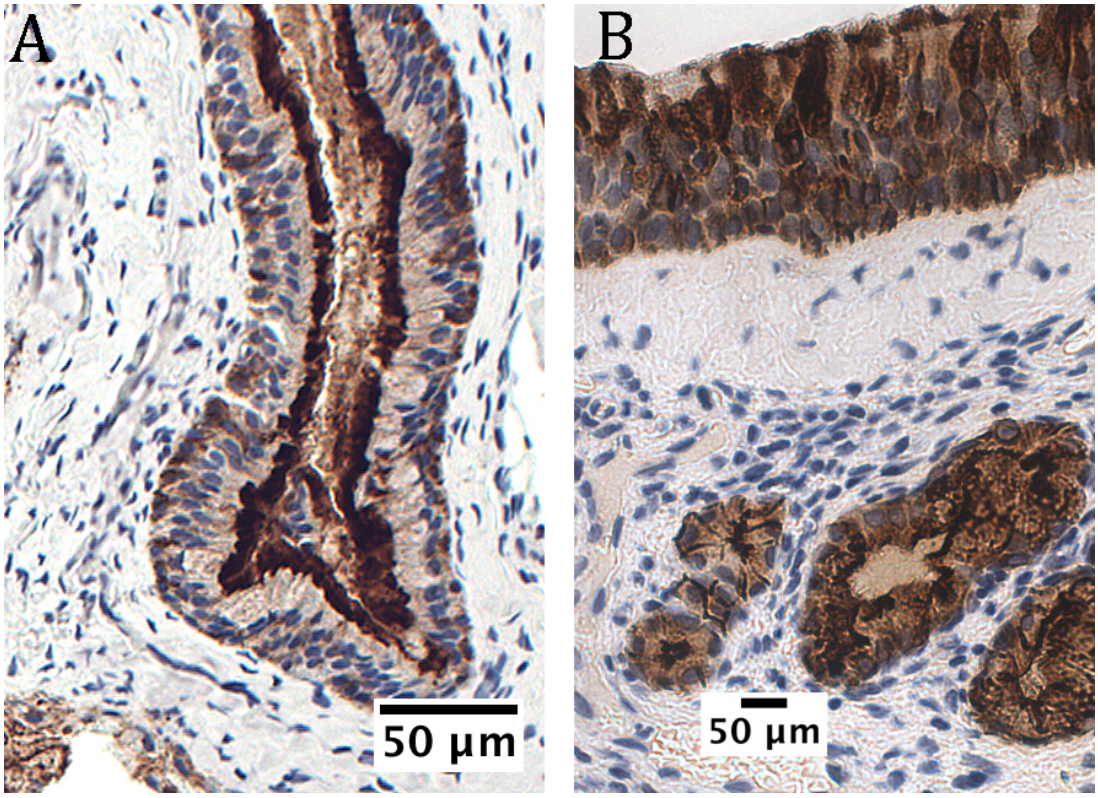

Figure 9.28: These photomicrographs demonstrate AQP5 localisation in surface epithelium in (A) normal sinonasal tissue and (B) CRSsNP sinonasal tissue. More intense localisation of AQP5 was seen in the cytoplasm of surface epithelial cells in CRSsNP tissue.

Figure 9.29: AQP5 intensity in glandular epithelium – Normal vs. CRSwNP

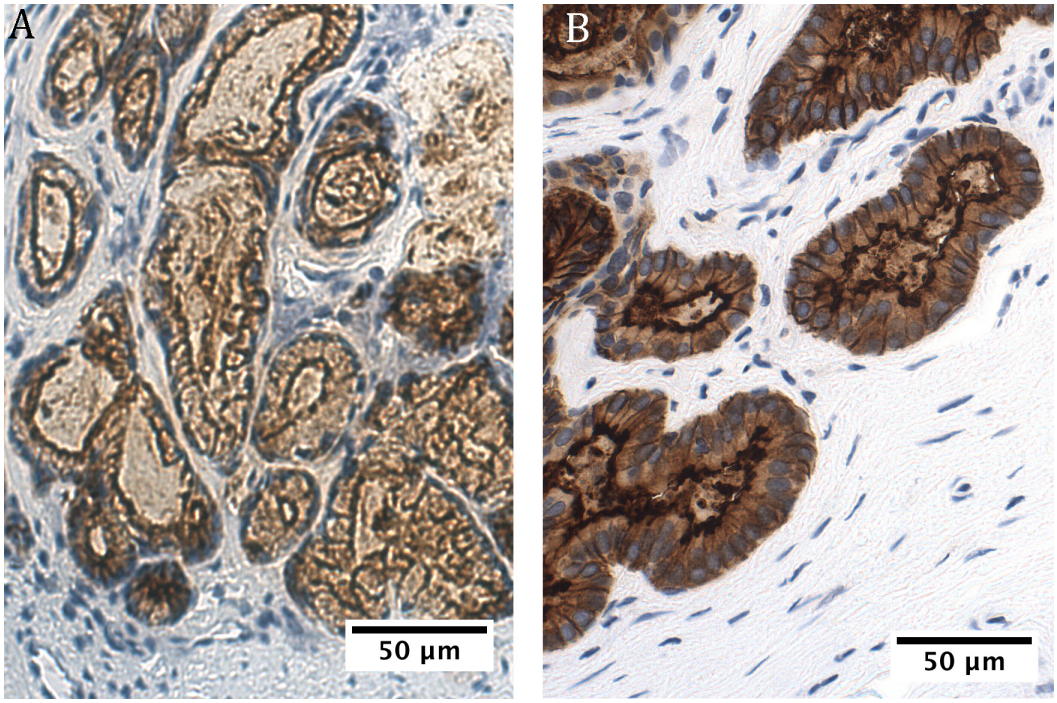

Figure 9.29: These photomicrographs demonstrate AQP5 localisation in glandular epithelium in (A) normal sinonasal tissue and (B) CRSwNP sinonasal tissue. More intense localisation of AQP5 was seen in the cytoplasm of glandular epithelial cells in CRSwNP tissue.

Chapter 10. Discussion

The aim of this research was to establish AQP expression profiles in normal sinonasal tissue and to test the hypothesis that there is a difference in AQP expression when comparing tissue from patients with chronic rhinosinusitis (CRS) (both with and without nasal polyps) to tissue from patients in the control group. The study has successfully characterised normal human sinonasal AQP mRNA expression and protein localisation. The findings correlate with the published literature and extend our knowledge of AQP expression in human sinonasal tissue by providing normal baseline AQP expression profiles for future reference. Furthermore, this study identified a difference in AQP localisation in both CRSwNP and CRSsNP tissue when compared to normal tissue for AQP4 and AQP5. Future investigation of AQP4 and AQP5 function in sinonasal tissue and how this differs in CRS may provide a better understanding of the aetiology of the condition, or these AQPs may become important targets in the targeted pharmacotherapy of CRS.

10. 1 Sinonasal AQP mRNA expression profiles

Identifying AQP mRNA in sinonasal tissue allows us to investigate if it is possible for AQP proteins to be produced within this tissue type. Sinonasal AQP expression profiles in normal human tissue were established at the mRNA level using highly sensitive quantitative real-time PCR.

Normal tissue

This study is the first to demonstrate the levels of mRNA expression of all known human aquaporins (AQPs) (AQP0–AQP11) in normal sinonasal tissue (Table 8.5). Additional analyses were performed to confirm the absence of AQP12b mRNA in normal sinonasal tissue. The AQPs present in sinonasal tissues at the highest mRNA concentrations were three of the four commonly identified as the key AQPs in lower airway studies (AQP1, AQP3 and AQP5) (Table 3.1).[60, 67, 79, 81-83, 120, 208] This study identified low levels of AQP4 in sinonasal tissue when, interestingly, it is known to be highly expressed in lower respiratory tissues. This finding was unexpected and difficult to interpret: it is possible that there are differences in the tissue architecture between the anatomical locations. However, the higher gland density in sinonasal

tissue and thicker mucosa in comparison to the lower airways would suggest an expected higher level of AQP4 in sinonasal tissue. Future studies with tissue harvested from sub-sites of mucosa using micro-dissection would allow for specific testing of mRNA expression in glandular or surface epithelium alone, rather than full thickness tissue as was used in this study. This would give a more precise idea of AQP mRNA expression in particular cell types and functional areas.

However, our findings were consistent with the few published studies of human sinonasal AQP mRNA. When this investigation commenced, no published studies of human AQP mRNA expression in sinonasal mucosa were identified in the literature, the one exception being a paper presenting a study of AQP3 and AQP4 mRNA in human nasal epithelial cell culture samples.[216] Jun et al. identified AQP3 and AQP4 mRNA expression (as well as mRNA of other ion channels) in human nasal epithelial cell culture samples taken from the inferior turbinate mucosa of 17 patients. Their study used degenerate RT-PCR methodology which aimed to compensate for degeneracy in genetic code between individuals. However, despite the difference in techniques between our current study and that of Jun et al., the identification of mRNA expression of AQP3 and AQP4 in normal sinonasal tissue is consistent with its expression in the nasal epithelial cell culture samples.

During the course of this study, two key studies of sinonasal AQP mRNA expression were published. Seno et al. investigated AQP1, AQP3, AQP4 and AQP5 mRNA expression in normal human inferior turbinate mucosa of six patients.[213] The RNA extraction system used by their study was a direct lysis method, which differs from the Trizol© organic solvent RNA extraction method used by our study. Most importantly, the PCR technique utilised by the Seno et al. study differs from that used by our study. While semi-quantitative reverse transcription PCR is able to confirm the presence of these AQP mRNAs, our study used real-time PCR which is highly sensitive and the current gold standard. They found mRNA for AQP1, AQP3 and AQP5; however, they were not able to identify AQP4 mRNA. Their findings of AQP1, AQP3 and AQP5 mRNA are consistent with this current study. However, this study also identified the presence of AQP4 mRNA. The highly sensitive quantitative RT-PCR technique used in this current study detected low levels of AQP4 mRNA which the semi-quantitative methodology used by Seno et al. would be less likely to detect, which could explain the discrepancy in AQP4 findings between these two investigations.

Subsequently, Shikani et al. published findings also identifying AQP5 mRNA expression in normal sphenoid or ethmoid sinus mucosa of five patients.[214] Their study used a commercial chloroform-based tissue lysis RNA extraction protocol that differs from the Trizol© organic solvent RNA extraction method used by our study. They used real-time PCR which was very similar to that used in our study. Despite the use of a potentially less sensitive assay, their finding of a relatively high expression level of AQP5 in sinonasal tissue is consistent with the findings in the current study.

It was important to successfully identify the mRNA expression of the AQPs known to be expressed in sinonasal tissue and the lower airway in our tissue samples. The reasons for its importance is that it confirmed that AQP1, AQP3, AQP4 and AQP5 protein was likely to be present in our tissues. (Prior to performing the immunohistochemical [IHC]part of the study, the techniques we used were effective in extracting RNA and identifying the presence of mRNA in sinonasal tissue, and that the tissue used in our study was similar to other tissue previously studied [both from upper and lower airways].)

In addition, the current study expands on the literature by demonstrating the presence of mRNA for an additional eight AQPs (AQP0, AQP2, AQP6, AQP7, AQP8, AQP9, AQP10 and AQP11). Many of these AQPs were found to be present at relatively low concentrations. The significance of this in terms of protein expression remains unknown and further work investigating AQP mRNA translation to protein and mRNA decay would be useful in establishing the extent of the role of these particular human AQPs.

Chronic rhinosinusitis (CRS)

Differences in AQP mRNA expression between normal and CRS tissue may suggest that difference in gene expression plays a role in the aetiology of CRS. Only two other published studies have investigated differences in the expression of AQP mRNA. Our study identified a significant difference in AQP expression profiles between CRSwNP tissue and normal sinonasal tissue. Significantly lower mRNA expression for AQP4 (1.2 fold reduction) and AQP11 (1.7 fold reduction) were identified in CRSwNP tissue (Figure 8.1) No other differences between normal and CRS tissues were identified for any AQPs.

These findings are consistent with those of Seno et al. who investigated AQP1, AQP3, AQP4 and AQP5 mRNA levels in a small cohort of normal and CRS

specimens (polyp status was not specified). They also found no statistically significant difference in the AQP1, AQP3 or AQP5 mRNA expression of tissues from eight CRS patients compared to six normal human sinonasal tissues.[213] It should be noted that they utilised semi-quantitative PCR as there are key differences in the sensitivity of quantifying the PCR product of a semi-quantitative methodology compared to the qRT-PCR methodology used in the current study. Our study also demonstrated no difference in mRNA expression of all other AQPs between normal and CRSwNP or CRSsNP tissue. This was not the expected finding of the study, and the significance of this finding may suggest that any difference in AQP protein expression or function is not likely to arise at the genetic level but more likely to be a protein translation, localisation or function alteration.

In contrast to our study, Shikani et al. identified approximately 10-fold less AQP5 mRNA in CRSwNP sinonasal mucosa specimens when compared to normal controls[214]. They concluded that there was likely to be lower global AQP5 expression in CRSwNP but that detailed protein studies would be beneficial to better contextualise this difference. The current study found no difference in AQP5 mRNA expression. Their study had similar power to our study (seven CRSwNP, seven CRSsNP and five normal controls), and they also employed real-time PCR. However, more detail on the normalisation of data in the Shikani study would be beneficial, as our study required complex investigation to identify a normalisation factor in the absence of a universally accepted control gene in sinonasal tissue. Further studies utilising larger patient numbers would also be helpful in resolving these conflicting findings.

In summary, this study extends the current knowledge by demonstrating AQP mRNA expression in diseased sinonasal tissue. Lower levels of mRNA expression of AQP4 and AQP11 were identified in CRSwNP specimens. It is possible that due to the very low mRNA concentration of these AQPs, the difference noted may be due to a number of factors: fast mRNA degradation, long protein half-life or a type 1 error.

10. 2 Sinonasal AQP protein expression profiles

The direct investigation of AQP protein by labelling with anti-AQP antibodies allows the understanding of where the proteins localise in sinonasal tissue, and whether they are on the surface and/or within particular cells. Identifying AQP mRNA allowed us to establish that it was possible for AQP0–AQP11 to be produced

in cells of sinonasal tissue; however, localising AQP protein confirms that the mRNA has been translated. While other techniques would be required to establish AQP functionality, immunohistochemistry allows us to observe the localisation of AQPs across tissue and to establish how and where the proteins are distributed, both of which have not been comprehensively studied in previous studies of sinonasal tissue. Using immunohistochemistry, our study successfully localised AQP1, AQP3, AQP4, AQP5, AQP7 and AQP11 in sinonasal tissue. The results are discussed in the following section based on anatomical sub-sites. The significance of intracellular AQP expression in CRS is also explored.

10. 2. 1 General findings

In our study AQP1, AQP3 and AQP5 were successfully localised in normal sinonasal tissue. AQP1 was identified in vasculature and connective tissue; AQP3 was strongly localised to the surface epithelium, as well as some expression in the glandular epithelium; while AQP5 was strongly expressed in the glandular epithelium, as well as being present in the surface epithelium. These sites are mostly consistent with published respiratory tract AQP literature (Table 3.1 and Table 3.2). AQP7 and AQP11 demonstrated low mRNA expression and we found low levels of AQP7 and AQP11 protein in cells of the surface and glandular epithelia, but only in some samples. The correlation of mRNA and protein levels is reassuring and suggests that these low levels could imply a highly specific role within nasal cells for AQP7 and AQP11, or a more generic physiological role for these AQPs across all human cells. Further studies investigating the localisation of AQP7 and AQP11 in other tissue types may shed more light on their role in sinonasal tissue.

AQP4 was also localised in our samples; however, the intensity of labelling was low and not consistent with other studies which was an unexpected finding. There were some difficulties with consistency of labelling in sinonasal tissue by the anti-AQP4 antibody. After comparison of our samples with published images[67, 83, 213, 324, 325] and discussion with a senior pathologist, we confirmed that this finding is not isolated to our study. AQP4 labelling is known to have limitations and there is some variability when investigating this protein using immunohistochemistry. Consequently, we should be cautious when drawing any conclusions regarding AQP4 localisation in sinonasal tissue, taking into account the technical difficulties experienced by all investigators in labelling AQP4.

10. 2. 2 AQP protein expression in glandular epithelium

AQP5

Our study identified the presence of AQP5 at the apical surface of normal sinonasal glandular epithelium. One of the normal functions of sinonasal glandular epithelium is to secrete the constituents of airway surface liquid (ASL) into the upper airway lumen. Apical epithelial expression of AQP5 has been identified as the rate-limiting barrier for water flow from glandular epithelium into the gland lumen in the lower airway, determining the hydration and viscosity of secretions (as discussed in Section 3.1.2).[120]

Our study is consistent with the published literature that reports AQP5 is typically expressed at the apical surface of normal glandular sinonasal epithelium.[67, 82, 213] Kreda et al. used *in situ* hybridisation and immunofluorescence when localising AQP proteins in normal human turbinate tissue (among multiple other sites within the airway).[82] In their study, two different anti-AQP5 antibodies were utilised, with both consistently and strongly reacting at the apical membrane of the glandular epithelium,. Seno et al. localised AQP5 in human sinonasal tissue samples using immunohistochemistry on frozen sections (note the different antigen retrieval techniques and anti-AQP antibodies sourced from Santa Cruz Biotechnology)[213] and found AQP5 was also localised to the apical surface of the glandular epithelium of sinonasal tissue. In a study of rat sinonasal tissue, Nielsen et al. used immunohistochemistry and immunoelectron microscopy to investigate AQP distribution in normal nasopharyngeal tissue finding clear localisation of AQP5 in the apex of glandular epithelial cells.[67] Also using rat mucosa, Song et al. localised AQP5 expression to the apical glandular epithelium of the nasopharynx and the rest of the respiratory tract.[120]

In contrast, the current study identified increased intracellular localisation of AQP5 in CRSwNP glandular epithelium. No other published study looking at sub-site localisation of AQP5 in CRS is available for comparison. Atypical and hyperplastic submucosal glands, and pseudocyst formation are key histological features observed in the glandular tissue of CRSwNP patients.[247, 326] Increased intracellular AQP5 may represent a response to the histological changes seen in CRSwNP tissue remodelling, or the AQP5 expression change may alter normal tissue remodelling regulation pathways and contribute to histological changes.

Confirming the alteration of AQ5 expression in the glandular epithelium of CRSwNP tissue in our study goes some way to supporting our hypothesis that AQPs may play a role in the production of the thickened secretions observed in CRS patients and is a key finding in our study. The possible significance of intracellular AQP expression was further explored in Section 10.3.

AQP4

AQP4 was not successfully localised in our normal sinonasal glandular epithelium. Our findings conflict with rodent studies that identified AQP4 at the basolateral surface of glandular epithelium[67, 83] Matsuzaki et al. investigated AQP localisation in normal mouse respiratory tract tissue, including the nasal cavity, using immunohistochemistry, immunofluorescence, immunoperoxidase and immunoelectron microscopy.[83] The study reported that AQP4 was localised to the basolateral surface of glandular epithelium in pseudostratified columnar epithelium of the upper airway (sample sub-sites were not specified). Nielsen et al. also successfully localised AQP4 to the basolateral surface of glandular epithelium in normal rat tissue.[67] There are several possible reasons for discrepancy between our findings and those of Matsuzaki and Nielsen. The reported limitations in drawing comparisons in AQP expression between rodents and humans should be taken into account.[218] In addition, immunohistochemistry, as a technique, can be influenced by many factors [327, 328] including differences in anti-body reactivity between species: different antibody interactions can also influence results when labelling target proteins with anti-AQP antibodies (or labelling any protein). Variability can exist between assays: it is difficult to determine that target protein is in fact not present, when lack of labelling by the antibody may simply demonstrate non-reactivity of the antibody used. Thus, it is possible that the technical limitations of our AQP4 investigation have prevented us from identifying true anatomical AQP4 localisation. Despite the limited localisation of AQP4 in normal glandular epithelium, this study identified greater intracellular AQP4 in CRSsNP glandular epithelium. The glandular epithelium of CRSsNP tissue is known to feature submucosal acinar cell hypertrophy and hyperplasia.[255, 329] It is not clear whether the higher expression of AQP4 noted by others[67, 83] is an artefact of acinar cell hypertrophy or a discrete physiological change. A better understanding of the role of AQP4 in sinonasal glandular function and performing a study using different anti-AQP4 antibodies would help to clarify the significance of this finding.

AQP3

AQP3 was identified in our study at the basolateral surface of normal sinonasal glandular epithelium which is consistent with other studies of the upper and lower respiratory tract.[67, 81-83] No changes in AQP3 were detected between normal and CRS tissues, implying that it is unlikely to have a role in the pathophysiology of CRS. No other published study looking at sub-site localisation of AQP3 in CRS is available for comparison.

Other AQPs

AQP1 was not detected in the glandular epithelium of any tissue in this study. AQP7 and AQP11 were localised to the cytoplasm of glandular epithelium in some CRSsNP and CRSwNP tissues; however, they were not present in normal tissue. The labelling for AQP7 and AQP11 was weak and inconsistent between samples, and thus, this is considered to be likely to be due to artefact. There are no other published studies available for comparison of these AQPs in sinonasal tissue.
Overall, our study confirmed AQP3 and AQP5 protein expression in the glandular epithelium of normal human sinonasal tissue, and localisation was consistent with previous sinonasal and respiratory tract studies. AQP3, AQP4 and AQP5 protein is present in CRS sinonasal tissue and localisation differences to normal tissue were seen in AQP4 and AQP5, suggesting a role in CRS aetiology or pathophysiology (see Section 10.4).

10. 2. 3 AQP protein expression in surface epithelium

AQP3

Our study of normal sinonasal tissue surface epithelium localised AQP3 in the apical and basolateral membranes and cytoplasm. Previous studies have localised AQP3 to the basolateral surface of this epithelium. (see Table 3.2 for comparison)[67, 82, 213, 217] The study by Seno et al. identified strong AQP3 localisation to the basolateral surface of surface epithelial cells in human tissue[213] and did not report any AQP3 expression at the apical surface. Similarly, the study of normal human nasal turbinate tissue by Kreda et al. localised AQP3 to the basolateral surface only.[82] In two rodent studies, AQP3 expression was investigated in normal rat nasopharyngeal tissue: these studies found AQP3 only at the basolateral surface of rat sinonasal tissue.[67, 217] The finding of AQP3 on the apical surface and cytoplasm of the surface epithelium

in normal human sinonasal tissue is novel. It is possible that the detailed nature of the immunohistochemical (IHC) scoring system in this study was more sensitive to the detection of apical and cytoplasmic AQP3 localisation. Future detailed protein localisation studies, such as labelling AQP proteins with immunogold electron microscopy, may provide confirmation of our observation.

No changes in AQP3 expression in CRS were identified in our study, implying it is unlikely to play a significant role in the pathophysiology of CRS: no other published study is available for comparison of AQP3 sub-site localisation in sinonasal disease. Previous AQP3 knockout studies in mice did not specifically detect major dysfunction in the upper respiratory tract.[209] However, AQP3 expression in all areas of sinonasal surface epithelial cells, as was found in our study, might suggest AQP3 plays some role in sinonasal homeostasis. Other studies have indicated that while AQP3 may not be fundamental or rate-limiting in water transport in this tissue, it does appear that AQP3 plays a part in pulmonary water transport mechanisms.[82, 209]

AQP4

In our study, low AQP4 expression levels were seen in normal tissue and AQP4 was only identified at the apex of the surface epithelial cells. This is in contrast to rat studies that found strong AQP4 expression at the basolateral surface epithelium [67, 217] Hypotheses (species differences, antibodies' specificity and low mRNA expression) to explain these findings have been mentioned in previous paragraphs; however, such low protein expression would suggest that AQP4 may have a limited role in sinonasal tissue homeostasis. No difference was detected in AQP4 expression at the surface epithelium of CRS tissue, and no other published study looking at sub-site localisation of AQP4 in CRS is available for comparison.

AQP5

In our study, AQP5 was found to have a similar distribution to AQP3 in normal tissue. It was localised to the apical and basolateral membranes and cytoplasm of surface epithelium. The immunofluorescence study by Kreda et al. reported apical AQP5 localisation in human nasal turbinate surface epithelium.[82] Also, Shakani et al. also used formalin-fixed, paraffin-blocked human sinonasal tissue for immunohistochemistry and the same Dako™ retrieval system as used in our study to investigate AQP5 expression in surface epithelium along with other epithelial proteins (E-cadherin, Septin-2).[214] Their study used different commercial anti-

AQP5 antibodies to those used in our study; however, the results of strong apical localisation in surface epithelium were consistent with our findings. No other published studies have identified basolateral or intracellular localisation of AQP5 at any other site in sinonasal surface epithelium.

In CRSsNP tissue, an increase in intracellular AQP5 expression was found in the surface epithelium. Given the lesser contribution of surface epithelial cells to the secretion of ASL components, it is not clear what role is played by apical AQP5 at the surface in the hydration of secretions when compared to glandular epithelium.[120] The apical and basolateral localisation seen here may indicate more of a role in complete transcellular water transport by AQP5, but functional studies would be required for better elucidation of this point. As this change has been observed in CRSsNP, a condition where epithelial hypertrophy and surface epithelial metaplasia are more common than in CRSwNP[247], it is possible that the changes in AQP5 here reflect altered cell-cycle regulation. As mentioned in Section 1.7: Additional features and functionality of mammalian AQPs, research continues into the role of AQPs in a range of features of normal tissue homeostasis including cell migration[39]; angiogenesis[52]; intercellular water transport[58]; and intracellular water transport in organelle function.[59] Improved understanding of regulatory pathways for these cell functions in the future will be of benefit when interpreting our findings. No other published study looking at sub-site localisation of AQP5 in CRS is available for comparison.

Other AQPs

AQP1 was not detected in the surface epithelium of any tissue in this study. AQP7 and AQP11 were localised to the cytoplasm of surface epithelium in some CRSsNP and CRSwNP tissues; however, they were not present in normal tissue. The labelling for AQP7 and AQP11 was weak and inconsistent between samples, and thus it must be considered possible that, although these AQPs were present, it may be due to artefact.

10. 2. 4 AQP protein expression in other sinonasal tissue sub-sites

AQP1 was localised in the vasculature of all tissue types, with no change observed in CRS. This is consistent with a broad literature base localising this AQP to vascular endothelium throughout the body.[5] AQP1 was also consistently localised to connective tissue, possibly expressed in the sub-epithelial lymphatics as is seen

elsewhere in the body.[5, 330] AQP4 was observed labelling connective tissue in some samples; however, this may be due to artefact from the technical challenges associated with anti-AQP4 antibodies. AQP7 was not localised to any tissue outside of the epithelia already discussed, and AQP11 was observed only to weakly label connective tissue in some samples.

10. 3 Significance of intracellular AQPs

Increased intracellular localisation of AQP4 and AQP5 proteins in CRS was a significant finding in this study. As discussed in Section 1.7.3, understanding of the significance of intracellular AQPs in normal tissue is an ongoing area of research.[7] Previous studies have identified several disease conditions in which AQPs were found to localise within cells, resulting in disruption of normal tissue homeostasis. These conditions include: AQP2 in nephrogenic diabetes incipidus[125, 126]; AQP4 in glioblastoma and cerebral oedema[17, 113-115]; and AQP5 in Sjogren's syndrome (discussed in Section 1.9).

The findings of our study have indicated increased intracellular AQP4 and AQP5 in two anatomical sub-sites within sinonasal tissue in CRS. We hypothesise several possible mechanisms for this:

- altered regulation of signalling pathways upstream to normal induction of AQP trafficking to the cell membrane,
- altered AQP structure leading to change in protein polarisation or key binding sites preventing insertion into the cell membrane,
- stimulation of endocytosis of AQP from the usual site of expression,
- increased or unregulated intracellular production of AQP,
- altered AQP expression or localisation of these AQPs at the membrane of intracellular organelles.

Increased intracellular AQP localisation in CRS may result from altered trafficking of the protein, leaving the AQP within the cell membrane rather than inserted in the usual membrane location. Regulation of AQP trafficking to the cell membrane is diverse and has been studied since AQPs were discovered in the 1990s; however, the understanding of the signalling pathways and regulation for each AQP remains incomplete (as mentioned in Section 1.5). [331] Mazzaferri et al. hypothesised that AQP4 basolateral trafficking from the site of manufacture in the Golgi apparatus is stimulated by phosphorylation but the signalling pathway is yet to be determined.[331] Their study used time-lapse imaging to determine that the transport of AQP4 protein

is non-linear within the cell and occurs along microtubules. As discussed in (Section 1.4), it is hypothesised that AQPs are processed in the Golgi apparatus prior to transport to their functional location within secretory vesicles. It is possible that interruption of the normal signalling pathway occurs in CRS, leading to failure of the AQP to insert at the usual location. An alternative explanation may arise from an interesting study by Carmosino et al. which showed that inflammatory stimulation of human gastric cells by exposure to histamine was found to increase intracellular AQP4 localisation.[332] AQP4 expressed at the basolateral epithelial cell membrane was observed to endocytose into intracellular vesicles after exposure to the inflammatory stimulus. The intravesicular AQP4 then underwent PKA-dependent phosphorylation but was not denatured. After histamine stimulation was removed, phosphorylation reversed and AQP4 proteins were returned to the basolateral cell membrane. Co-labelling and serial photography confirmed that the proteins were not transported to organelles but remained in vesicles until they were returned to the cell membrane. In this way, the inflammatory cytokine-mediated stimulation of sinonasal mucosa in CRS may drive endocytosis of AQP4 and/or AQP5 and offer an explanation of our study's experimental findings of increased cytoplasmic AQP expression.

Increased intracellular AQP5 expression was also observed in the glandular epithelial cells of salivary glands of patients with Sjogren's syndrome.[124] A recent study identified the muscarinic type 3 receptor in salivary glands as key to the suppression of AQP5 trafficking to the cell membrane in Sjogren's syndrome[333] An increase of the same AQP in an equivalent histological secretory tissue gives rise to the possibility of a common defect in AQP5 trafficking between CRS and Sjogren'syndrome. The same muscarinic receptor is known to be expressed in sinonasal tissue[334]; however, the underlying pathogenesis of Sjorgren's syndrome is understood to be autoimmune, and CRS is essentially believed to arise from an altered host vs. environment response.[220] This hypothesis represents an interesting avenue for future study.

Overall, this study has identified several instances of increased intracellular AQP localisation: further studies will be required to identify the driver(s) of this change in CRS.

10. 4 Significance of altered AQPs in CRS

This study identified altered protein expression of AQP4 and AQP5 in CRS. Alteration of each of these AQPs has been identified in other disease examples involving the disruption of normal water transport, as mentioned in Section 1.9.

10. 4. 1 AQP4 in CRS

While our study was not able to identify strong AQP4 expression in normal tissue, several other studies have localised AQP4 to the basolateral surface of sinonasal glandular epithelium.[67, 213] Seno et al.'study is the only other study of AQP4 in human CRS tissue, and they were unable to identify a difference in expression between normal tissue and that of CRS.[213]} In our study, AQP4 was difficult to label consistently in normal tissue; however, strong , convincing labelling of AQP4 protein was seen in the cytoplasm of CRSwNP glandular epithelium.

By considering the literature that describes changes in AQP4 expression in cerebral oedema, we can draw parallels with the pathological features of CRS, especially sinonasal mucosal oedema. Section 1.9.1: Cerebral oedema discussed increased cytotoxic and vasogenic cerebral oedema seen in AQP4-null mice.[112-114] In the brain, AQP4 is principally localised to the perivascular astrocyte endfeet[335] where astrocytes make up the bulk of connective tissue as well as forming the blood-brain barrier (BBB). AQP4 is key in BBB water transport as well as cell adhesion and neuroinflammation.[287] AQP4 function in cerebral oedema is disrupted in BBB breakdown caused in part by MMPs (as previously mentioned in Section 4.4.3: MMPs and AQPs). After activation of MMP-3 and MMP-9 by inflammatory cytokines and the resulting destruction of local structural proteins, oedema results from the alteration of the interface between the astrocytes and the vascular endothelium and the altered polarisation of AQP4 at the astrocyte membrane.[336] (discussed further in Section 1.9.1: Cerebral oedema) Wolburg et al. also identified the interruption of AQP4 localisation due to degradation of local proteins by MMPs that assist in keeping AQP4 in the BBB in its stable orthogonal array structure, resulting in oedema.[115]It is possible that similar disruption of normal AQP4 function in sinonasal glandular epithelium could occur in CRS. No previous studies have identified the alteration of AQP4 in CRS or other sinus disease. Alteration of AQP4 at the basolateral surface of sinonasal glandular epithelium would change the normal water flow from the vasculature into the glandular epithelium, which is analogous to the changes at the BBB in cerebral oedema. This might lead to interruption of AQP4

regulatory pathways involving cAMP or protein kinases; however, we suspect MMPs and local tissue architecture will play the most significant role.[86, 87] A case for MMPs in CRS has been made in Section 4.4.2: MMPs and we hypothesise that the alteration of MMP activity previously observed in CRSwNP tissue is associated with altered AQP4 localisation in CRSwNP tissue. Future study in this area to clarify this is required.

10. 4. 2 AQP5 in CRS

As raised in Section 4.5.2: Studies of AQPs in CRS, several studies have identified some form of alteration of AQP5 expression in CRS.[213, 214, 312, 313] While the study by Seno et al. has been discussed at length and has methodology very similar to this study, they were unable to identify a statistically significant difference in AQP5 expression between normal and CRS tissue.[213] Shikani et al. found higher AQP5 mRNA expression in CRS but failed to detect a correlating difference in protein expression.[214] Huang et al. found that Chinese polyp tissue had lower AQP5 mRNA expression than normal tissue of different ethnicity[313]. However, this may have been due to the decreased concentration of surface or glandular epithelial structures within a polyp when compared to normal or CRS sinonasal mucosa, thus leaving this result difficult to interpret. Finally, Jiang et al. found lower AQP5 protein in the surface and glandular epithelium of CRS tissue when compared to that of allergic rhinitis.[312] In CRS, there is no 'normal polyp' to act as a control and these authors did not compare their findings to normal sinonasal tissue. Overall, this study is the first published study to identify statistically significant higher AQP5 expression in CRS: future study of the role played by AQP5 in water transport and the remodelling in sinonasal tissue that develops polyps will aid the understanding of the significance of our findings.

As previously raised, AQP5 was identified as the rate-limiter in secretion hydration through studies that investigated the pathogenesis of Sjogren's syndrome.[118-121] When inserted at the apical membrane of glandular epithelium, AQP5 functions to move water from within the glandular epithelial cells into the lumen of the gland. When AQP5 is present at another location (the basolateral surface of the same cells), the secretions are thickened and concentrated: this has been shown to be the case in Sjogren's syndrome (as discussed in an earlier section).[80] Parallels between Sjogren's syndrome and CRS have been explored earlier (Section 1.9.2): they have, in common, the hyper-concentration of secretions with relative dehydration of the

ASL produced by glands. Increased intracellular AQP5 suggests an alteration of the function of AQP5 at the apical gland epithelium, although functional studies are required to address this additional hypothesis. The thickened, dehydrated luminal secretions suggest that even if apical AQP5 is functional, less water is being effectively transported from the interstitum, through the cell and out into the ASL in the gland lumen. It is this change that we propose plays a part in the pathological mechanism for CRS.

10. 5 Study limitations

10. 5. 1 Study samples

The patient groups in this study were similar with no statistically significant difference in age at surgery or severity of CRS (on the CT scan). Sex distribution between groups was not similar; however, major studies of CRS patient populations do not report a sex-related difference in CRS prevalence [220, 337] so it is unlikely that these differences in sex distribution have biased our results.

The possible allocation of patients with mild polyps to the CRSsNP patient group was a theoretical source of error in this study. Patients with significant medical co-morbidities that may impact on the study (e.g. cystic fibrosis) were excluded at the study recruitment stage. Normal control patients undergoing unrelated neurosurgery were excluded from recruitment if history, examination or pre-operative CT scan suggested CRS or any other sinonasal condition. CRSwNP patients were readily identifiable and records were reviewed to confirm if polyps had been identified at consultation or surgery, or both. For CRSsNP patients, careful review of their consultation record, pre-operative CT report and operation note was undertaken to reduce the likelihood of a patient with polyps being incorrectly allocated to the CRSsNP group. Inclusion of patient tissue in the wrong experimental group would be an important source of error and would skew the observations of AQP protein and mRNA expression profiles.

10. 5. 2 PCR method

This study used full thickness sinonasal specimens for qRT-PCR which has some limitations. The tissue is homogenised during RNA extraction and the experimental result reflects that mRNA is present across all cell types from all structures within the tissue sample. More specific information may be obtained with future studies by

micro-dissection of the sinonasal surface or glandular epithelia to investigate AQP mRNA expression within particular cells in sinonasal tissue.

The inherent histological differences between normal and CRS tissues (e.g. epithelial hypertrophy or thinning, squamous metaplasia, glandular hypertrophy, fibrosis, pseudocysts and the presence of increased numbers of inflammatory cells[222, 247]) should be taken into account when interpreting differences in mRNA expression between groups. While the total AQP mRNA concentration in the tissue samples assessed may not be altered between groups, there may be differences in AQP mRNA expression at specific sub-sites within the tissue that could not be detected using this technique.

Quantitative PCR is a very sensitive and specific technique for identifying mRNA products and will detect even low levels of mRNA. In this study, some of the AQP mRNA concentrations detected were very low (a relative concentration of <0.005). It should be noted that when comparing expression between normal and CRS tissues, the relatively very low mRNA concentrations of some AQPs may give rise to a type I error (i.e. for AQP4 and AQP11 in CRSwNP) or a type II error (for the other AQPs). Future studies with increased power would help to address this issue.

It is possible that such a low concentration of mRNA suggests little functional requirement from the cell for the protein product; however, it should be kept in mind that mRNA levels and protein levels do not necessarily correlate directly.[338, 339] Due to multiple steps that regulate protein production from mRNA transcription through to protein degradation, the abundance of protein produced from mRNA is widely variable. For this reason, the combination of PCR with protein study (e.g. immunohistochemistry as in this study) is an important step in understanding AQP expression in sinonasal tissue.

10. 5. 3 IHC method

The optimisation of the anti-AQP antibodies and the inclusion of positive and negative control tissues (see Appendix) resulted in the successful labelling of AQP1, AQP3, AQP5, AQP7 and AQP11 in the study samples. As mentioned, AQP4 is notoriously difficult to label with anti-AQP4 antibodies and, as such, interpretation of our finding in CRSwNP glandular epithelium must take this limitation into account. The failure to successfully optimise the anti-AQP10 antibodies could be addressed by selecting another commercial antibody for trial, sourcing antibodies

directly from another institution that has successfully studied this AQP or utilisation of alternative retrieval techniques to those used in this study.

The choice of scoring methodology has impacted on the interpretation of the intensity of AQP localisation in the sinonasal mucosa sub-sites. The score was based on the intensity of anti-AQP labelling at each sub-site, and was not based on the intensity across the whole slide. This decision was made to decrease the impact of the inherent histological differences between normal and CRS tissues which have been previously mentioned. The scorer was also able to exclude the influence of leukocytes present in CRSwNP and CRSsNP, which labelled strongly for AQP3. It is not clear if other published studies took this into account when assessing the intensity of AQP labelling: this may be a reason for discrepancy between findings. The investigation of AQP expression in the inflammatory cells that infiltrate sinonasal tissue in CRS could be an area of future study.

Finally, in reviewing the methodology, it was noted that to be able to cut sections of sinonasal tissue in which fragments of ethmoid bone were present, decalcification of the paraffin-blocked sinonasal tissue block was required. This was performed on all tissue blocks to standardise experimental conditions. It is known that these methods sometimes fragment and cause the degradation of RNA in tissues[340, 341]: comparative studies between decalcified and non-decalcified tissue would be required to establish if this was an issue in this study.

10. 6 Future research

This study provides baseline AQP expression profiles for future research into AQPs in sinonasal tissue. Future research could branch into several interesting areas which, in normal tissue, could include: functional studies of AQPs in sinonasal mucosa including water transport and cell-cycle control; regulation of AQPs in sinonasal mucosa; and interaction/co-location/co-regulation of AQPs and MMPs. The specific study of water movement across the highly vascular sinonasal tissue would need to be multifactorial and should include, but not be limited to, AQPs. Building a working model of water transport across sinonasal mucosa would be an important step towards better understanding sinus disease and CRS. However, this complex system would need to take into account all of the water transport mechanisms mentioned in Section 2.4, as well as a more detailed understanding of AQP function and regulation in sinonasal mucosa and in secretion production.

An alternative methodology to localise AQP proteins with more precision should be considered, including other imaging or labelling techniques. Immunogold electron microscopy is a method that would facilitate more detailed observation of intracellular AQP localisation in particular, and may shed more light on the role of AQPs in normal cell homeostasis.

Finally in CRS, studies of the role of AQPs in tissue remodelling would be of benefit, and could include comparison of CRS sinonasal tissue, pseudocysts and mature polyps. This may shed light on how normal tissue remodelling becomes altered and polyps develop. A better understanding of tissue remodelling would allow the development of targeted pharmacotherapy in early polyp formation and tissue remodelling, and has the potential to revolutionise the management of CRS for patients.

10. 7 Conclusion

In conclusion, this study posed the questions: are human AQPs expressed in normal sinonasal mucosa, and is there a difference in their expression in CRS tissue? The study successfully characterised the normal human sinonasal mRNA expression of AQP0–AQP11, with lower AQP4 and AQP11 mRNA expression identified in CRSwNP. The study also localised normal protein expression of AQP1, AQP3, AQP4, AQP5, AQP7 and AQP11, and identified increased intracellular localisation of AQP4 and AQP5 in both types of CRS. This work was mainly consistent with published AQP literature, and contributes baseline knowledge of sinonasal AQP expression for future studies in sinonasal mucosa and in CRS. These findings establish a role for AQPs in CRS pathophysiology and they may prove to be key targets in developing treatment for this chronic condition.

Appendix: Immunohistochemistry control tissues

Figure A.1: Immunohistochemical positive controls

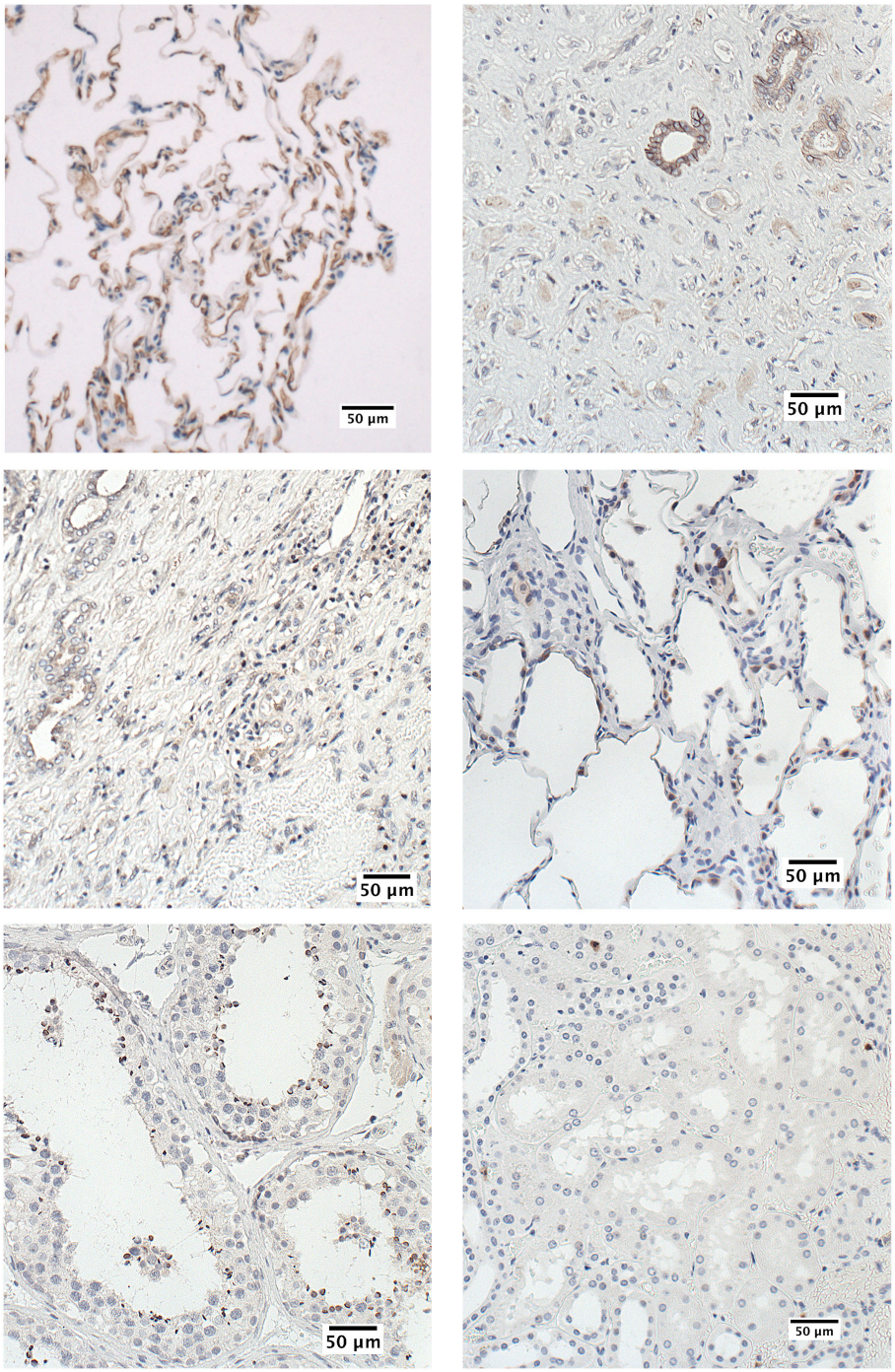

Figure A.1: Photomicrographs of positive control tissue for each anti-AQP antibody (10x magnification): (A) AQP1 – lung; (B) AQP3 – kidney; (C) AQP4 – kidney; (D) AQP5 – lung; (E) AQP7 – testis; and (F) AQP11 – kidney.

Figure A.2: Immunohistochemical negative controls

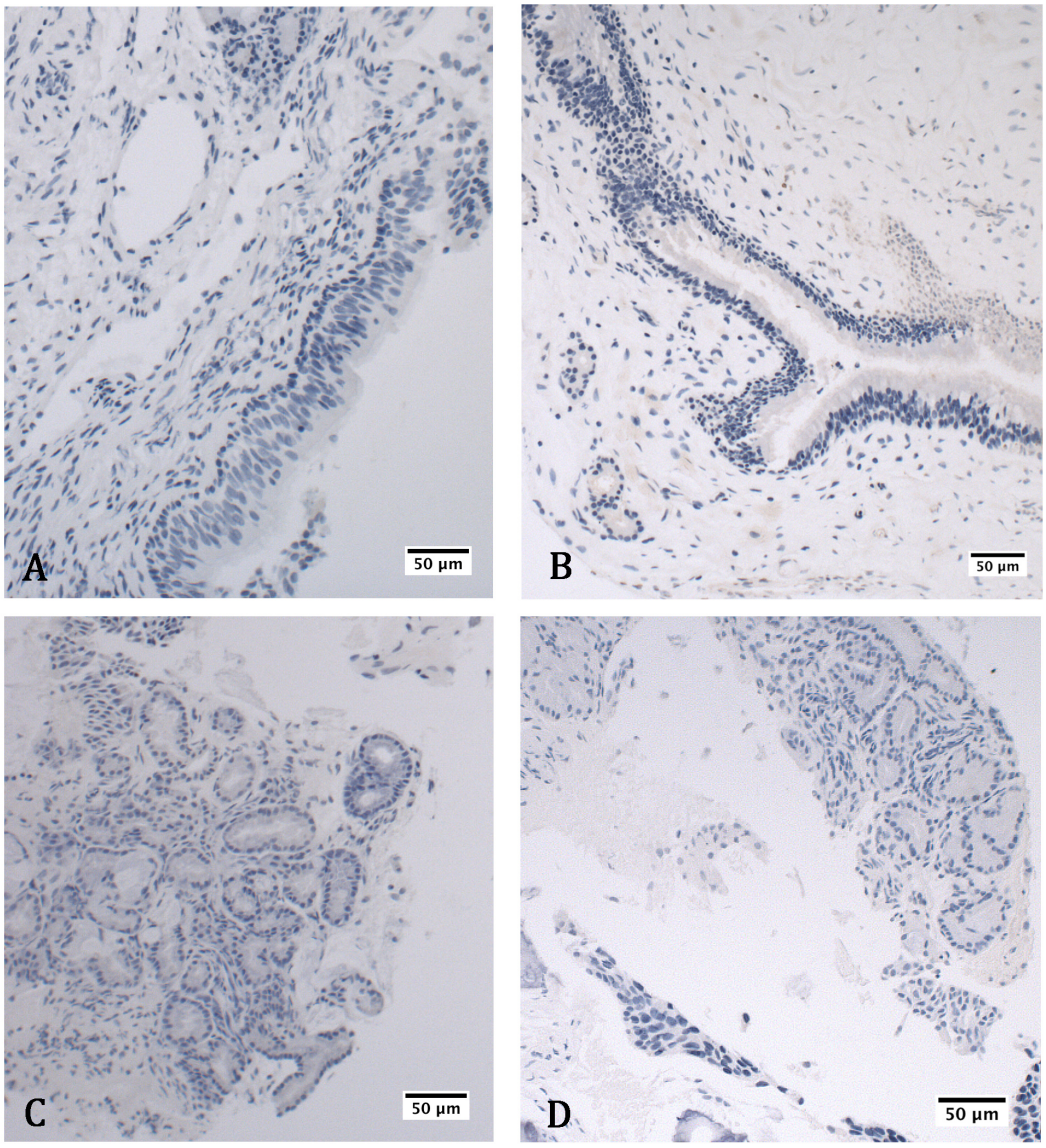

Figure A.2: Photomicrographs of immunohistochemistry negative control samples, confirming that the antibody detection system did not falsely label tissue undergoing each retrieval technique (all sinonasal mucosa, 10x magnification).
Retrieval techniques: (A) alkaline; (B) trypsin; (C) no retrieval and (D) citrate.

Appendix: Publication

Aquaporin expression profiles in normal sinonasal mucosa and chronic rhinosinusitis

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Key Words: Rhinitis, sinusitis, aquaporin, nasal polyps, paranasal sinuses, nasal mucosa, immunohistochemistry, PCR

Abstract

Introduction

Thickened secretions, mucosal edema, and polyp formation are pathological features in chronic rhinosinusitis (CRS) that could theoretically be caused by aberrant water flow through sinonasal mucosa. Aquaporins (AQP) are a family of proteins with roles in water transport, with tissue specific expression profiles. This study aims to determine if AQP expression in sinonasal mucosa is different between normal controls and patients with CRS, either with (CRSwNP) or without (CRSsNP) nasal polyps.

Methods

During endoscopic sinus surgery or trans-sphenoidal surgery sinonasal tissue was collected and classified as CRSwNP (n=13), CRSsNP (n=10) or normal (n=10). mRNA expression of human AQP0–AQP12b was determined using quantitative real-time PCR. Cellular localization of AQP1, AQP3, AQP4, AQP5, AQP7 and AQP11 was determined by immunohistochemistry.

Results

mRNA of AQP0-AQP11 was identified in all samples. AQP12b mRNA was not detected. Significant differences in the mRNA expression levels of AQP4 and AQP11 were identified between normal and CRSwNP patients ($p<0.05$). Differences in the cellular localization of AQPs were observed in both CRSsNP and CRSwNP patients vs. normal controls. More intense localization to the cell cytoplasm was observed for AQP5 in glandular epithelium (CRSwNP; $p<0.05$) and surface epithelium (CRSsNP; $p<0.05$), and AQP4 in glandular epithelium (CRSsNP; $p<0.05$).

Conclusion

This study characterized AQP mRNA expression and protein localization in normal human sinonasal tissue. Significant differences in mRNA expression were found for AQP4 and

AQP11 in CRSwNP and differences in protein localization patterns of AQP4 and AQP5 were identified in both types of CRS.

Introduction

In chronic rhinosinusitis (CRS) viscous secretions, edematous sinonasal mucosa, and formation of polyps are salient pathological findings, [1, 2] and could theoretically be a result of altered water transport through diseased sinonasal tissue. The study of water movement across normal sinonasal epithelium has been limited and the potential for a role for altered water transport in CRS has not been explored. Water transport at an epithelial level is complex and incompletely understood. However, local epithelial cell membrane water channel expression is known to influence tissue water permeability. [3] Aquaporins (AQPs) are a family of cell membrane water channel proteins. Originally discovered in the 1990s, 13 mammalian AQPs have now been identified (AQP0-AQP12). [3-5] AQPs transport water across cell membranes, with some AQPs also transporting glycerol and other small, inert molecules.[6, 7] They are found throughout the body and are key to trans-epithelial water transport. Importantly, alteration in AQP expression has been implicated in a range of diseases elsewhere in the body. [6, 8-11]

The literature is limited regarding AQP expression in human sinonasal tissue (non-olfactory),[12-17] with no published study characterizing baseline expression of all 13 known human AQPs. The potential exists for altered AQP expression to disrupt water flow through epithelial tissues and contribute to disease. [18] An AQP5 gene knock-out mouse model produced airway secretions that were significantly more concentrated and viscous than normal, mimicking the thickened mucus lining the airways of CRS patients.[18] We hypothesize that several of the histological changes present in CRS diseased tissue may be influenced by altered AQP expression: thickened secretions, sub-epithelial edema and pseudocyst development - a precursor of polyp formation.[19-21] This study aims to determine if AQP expression in sinonasal mucosa is different between normal control tissue and patients with CRS, both with (CRSwNP) and without (CRSsNP) nasal polyps.

Methods

Tissue samples

This study was approved by the Southern Adelaide Clinical Human Research Ethics Committee with informed consent obtained from all patients. Ethmoidal infundibular mucosa was obtained from patients undergoing endoscopic sinus surgery for CRSsNP and CRSwNP, as defined by EPOS 2012[22]. Exclusion criteria were revision surgery, endoscopic sinus surgery for a clinical indication other than CRS and significant comorbidities (e.g. cystic fibrosis). Normal control tissue was collected from patients undergoing endoscopic trans-sphenoidal pituitary surgery without CRS symptoms or CT scan evidence of sinus disease . Following harvest, tissue samples were immediately placed into RNAlater™ (Ambion, USA) at -20°C and 10% formalin.

RNA extraction, quality control & cDNA synthesis

RNA was extracted from 0.025g of each sample (n=35) using a modified TRIzol® protocol, as previously described by Woods et al. [23] The protocol was modified to include a high salt precipitation step recommended by the manufacturer in tissues with proteoglycans and/or polysaccharides. RNA concentrations were determined (NanoDrop 8000 Spectrophotometer ThermoScientific, Delaware, USA) and RNA quality was assessed (2100 Bioanalyzer™; Agilent®, United States). Samples with degraded RNA (RNA Integrity Number <4.0 or review of digital gel electrophoresis image; n=3) were excluded from further analysis.[24] The remaining patient samples (n=32) included in the study were: 9 control, 10 CRSsNP and 13 CRSwNP.

1µg of RNA underwent single step elimination of contaminating genomic DNA and reverse transcription using the RT^2 First Strand ™ kit (Qiagen, Germany) following the manufacturers protocols. Two negative controls from each patient group and an ultra pure water PCR control were included during cDNA synthesis.

Real-time quantitative PCR & data normalization

Commercial RT^2 qPCR Primer™ assays were used (Qiagen, Germany) to assess mRNA gene expression of 13 Aquaporin genes (AQP0-12b): AQP0 (MIP) – Catalogue number PPD04546A, AQP1 - PPH59997A, AQP2 - PPH00017A, AQP3 - PPH14747A, AQP4 - PPH05944B, AQP5 - PPH16382A, AQP6 - PPH09464A, AQP7 - PPH22164B, AQP8 -

PPH13652A, AQP9 - PPH05945A, AQP10 - PPH15277A, AQP11 - PPH16785A, AQP12b - PPH60610A. No assay for AQP12a was available.

Each sample was assayed in triplicate using RT^2 SYBR Green ROX FAST Mastermix (Qiagen, Germany). A genomic DNA positive control and an ultra pure water negative PCR control were included for each assay. AQP PCR conditions consisted of initial enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 30 seconds, then final hold from 65°C to 95 °C for melt curve analysis, in the Rotorgene 6000 cycler (Corbett Research, Australia).

In the absence of a universal control gene for sinus tissue 7 housekeeping genes were assessed for normalization: 18s rRNA, and HPRT, UBC, Beta-Actin, PBGD, GAPD, and ribosomal protein L32. Commercial RT^2 qPCR Primer™ assays were used to assess 18 SrRNA (Catalogue number PPH05666E) and HPRT (PPH01018C)(Qiagen, Germany), prepared as described above. Primer sequences for the remaining 5 genes are: UBC: forward primer ATTTGGGTCGCGGTTCTTG, reverse primer TGCCTTGACATTCTCGATGGT; β-Actin: forward GCCGATCCACACGGAGTACT, reverse TTGCCGACACGATGCAGAAG; PBGD: forward CTTTCCAAGCGGAGCCATGTCTGG, reverse CATGAGGGTTTTCCCGCTTGCAGA; GAPD: forward TGCACCACCAACTGCTTAGC, reverse GGCATGGACTGTGGTCATGAG; L32: forward TTCCTGGTCCACAACGTCAAG, reverse TTGTGAGCGATCTCGGCAC. Each of these housekeeping gene assays comprised of 10 uL of 2XQuantitect SYBR green mastermix, 2uL forward gene-specific primer, 2uL reverse gene-specific primer and 6uL of cDNA sample. [25] The geometric mean of multiple housekeeping genes was employed for normalization.[26] The GeNorm algorithm was used to select the three most stably expressed housekeeping genes observed in the sinus tissue samples.[26] The geometric mean of HPRT, UBC and Beta-Actin was used to normalize the AQP assay results.[26]

Immunohistochemistry

AQPs known to be expressed in the lower respiratory tract were selected for immunohistochemical study (AQP1, AQP3, AQP4 and AQP5) [12] [27] in addition to AQP7 and AQP11. Some control samples did not include tissue suitable for immunohistochemistry and were excluded, reducing sample numbers to n=28 (5 control, 13 CRSwNP, and 10 CRSsNP).

Tissue was paraffin fixed, 4μm paraffin sections were cut, deparaffinized, rehydrated, then quenched with 1% hydrogen peroxide in 50% ethanol. The sections were washed with a

Tris/Salt solution (pH 7.6), applicable antigen retrieval performed and commercial anti-aquaporin antibodies (Table 1) were diluted to required concentrations with antibody diluent (10% normal goat serum, Sigma, G9023-10mL)(Table 1). 200µL of antibody was applied to each sample and incubated overnight at 4°C. The DakoCytomation EnVision™ Dual Link detection system (Dako, Denmark) was used following the manufacturer's protocol, then sections were counterstained with haemotoxalin and lithium carbonate before washing and dehydration using xylene rinses. Coverslips were applied using DePeX (VWR international, Pennsylvania, USA).

Sections were viewed on an Olympus BX 50 Brightfield Photomicroscope ™ equipped with Q-Imaging MicroPublisher ™ RTV 5 megapixel digital camera and Q-Capture software© (Q-Imaging, Canada). Positive controls for each anti-aquaporin antibody were cross-referenced with published images, confirming appropriate AQP labeling. [13, 16, 28-30][31, 32] A semi-quantitative 6-point Likert scale was used to score intensity of antibody labeling (-, +/-, +, ++, +++, or "not present") for a number of histological sub-sites (Table 2). Samples with a score of "not present" were excluded from sub-site statistical analysis. Areas of tissue demonstrating salient findings were photographed for reference (images processed using ImageJ™, U. S. National Institutes of Health).

Statistical analysis

The normalized relative concentrations of each sample for all AQP qRT-PCR assays, and the immunohistochemical labeling intensity scores for AQPs at each anatomical location were analyzed using PRISM© Version 6.0c (GraphPad Software, Inc). To determine if there was any difference in expression for an AQP between disease groups (normal vs. CRSwNP vs. CRSsNP) a Kruskal Wallis analysis was initially performed, followed by Dunn's test for multiple comparisons. $p<0.05$ was considered statistically significant.

Results

Expression of human AQPs in normal sinonasal mucosa

Quantitative real-time PCR successfully identified mRNA for 12 of the 13 known mammalian AQPs in normal human sinonasal tissue, with a wide range of relative concentrations (Figure 1, Table 3). No AQP12b mRNA was detected. High resolution melt profiles of genomic DNA controls were compared with the manufacturer's data for quality control, demonstrating functionality of all assays including AQP12b.

Immunohistochemistry localized AQP1, AQP3, AQP4, AQP5, AQP7 and AQP11 in normal human sinonasal mucosa (Table 2, Figure 2). AQP1 localized to vasculature and connective tissue. AQP3 localized strongly at the basolateral membrane of both surface and glandular epithelium, less strongly at the apical membrane and in the cell cytoplasm. AQP4 localized weakly to the apical membrane of the surface epithelium and to connective tissue. Strong localization of AQP5 at the apical and basolateral membranes of the surface epithelium was observed, with less intense AQP5 localization in the cytoplasm of these cells. AQP5 also strongly localized to the apical membrane of the glandular epithelium, with less intensity within the cell cytoplasm and the basolateral membrane. Low levels of AQP7 and AQP11 localized to the cytoplasm of the surface and glandular epithelium, but this was inconsistent between specimens.

AQP mRNA expression in CRS

Analysis of AQP expression comparing normal sinonasal tissue to CRS disease identified lower mRNA expression of two AQPs (Figure 1): AQP4 was 1.7-fold less (CRSwNP, $p<0.01$) and AQP11 was 1.2-fold less (CRSwNP, $p<0.05$). mRNA expression of the remaining AQPs was not significantly different between control and CRS disease, with or without polyps ($p>0.05$). It was noted that differences in mRNA expression approached significance for AQP3 (KW p=0.0519), AQP7 (KW p=0.0680) and AQP10 (KW p=0.0866).

AQP protein expression in CRS

Differences in localization intensity of AQPs were identified in normal compared to CRS diseased tissues (Table 2, Figure 2, Figure 3). Localization of AQP4 was greater in the cytoplasm of glandular epithelial cells in CRSsNP (++ compared to +, $p<0.05$), and localization of AQP5 was greater in the cytoplasm of both the surface epithelium in CRSsNP,

and in glandular epithelial cells in CRSwNP (both ++ to +, $p<0.05$) when compared to control tissue.

Discussion

This is the first comprehensive study of the 13 mammalian AQPs in human sinonasal tissue. It provides increased knowledge of AQP mRNA expression and protein localization patterns in the upper airway and identified differences in AQP expression that may suggest a possible role for these proteins in CRS pathogenesis. The data provides a basis for future investigation of trans-epithelial water transport via AQPs in sinonasal mucosa in health and disease.

AQPs in normal sinonasal mucosa

This study is the first to demonstrate mRNA expression of AQP0-AQP11 in normal sinonasal tissue. The AQPs present in sinonasal tissues at the highest levels (AQP1, AQP3 and AQP5) are those commonly identified as the key AQPs in lower airway studies, with the exception of AQP4.[12] These findings are consistent with the few published studies assessing AQP mRNA expression in normal sinonasal tissue,[16] nasal epithelial cell culture [14] or nasal tissue biopsies[17], which all used semi-quantitative PCR. This study expands on the current literature demonstrating the presence of mRNA for an additional 8 AQPs (AQP0, AQP2, AQP6, AQP7, AQP8, AQP9, AQP10, AQP11). Many of these are present at quite low relative concentrations, and the significance of this remains unknown as rates of mRNA translation into protein and mRNA decay have yet to be studied for the different AQPs. It should be noted that some of these AQPs may be present in a specialized location or a specific compartment of the sinonasal mucosa, resulting in apparent low-level expression when assessed as part of a full- thickness mucosal biopsy.

AQP proteins have specific expression patterns within cells of a particular structure, with specific AQPs being incorporated in the apical, the basolateral cell membrane, or both. This study investigated the cellular localization of AQPs in normal sinonasal tissue. Using comparative immunohistochemical techniques, the results and localization of AQP1, AQP3 and AQP5 are in agreement with other published studies of AQP protein expression in human upper respiratory tract [12] [16, 17].

Investigation of protein expression of additional AQPs with low mRNA expression (AQP7 and AQP11) showed very low-levels of protein in cells of the surface and glandular epithelia, but only in some samples. This correlates with the mRNA data and suggests that little protein

is translated from the AQPs with lowly expressed mRNAs, thereby questioning their functional significance in the sinuses. Overall, our findings of AQP expression in normal sinonasal tissue are consistent with the literature for both upper and lower airways, with the exception of AQP4.[10]

AQPs in CRS

Small but significant differences in mRNA expression for AQP4 and AQP11 (1.2 and 1.7 fold less) were identified in CRSwNP tissues. However, this did not correlate with lower protein expression, thus questioning the functional significance of the mRNA findings. However, this may be related to protein half-life. Our data suggests that changes to AQP transcription are not altered in the setting of CRS, but does not exclude potential changes to translation and AQP protein functionality. Another study also failed to demonstrate a statistically significant alteration in AQP1, AQP3, or AQP5 mRNA expression in CRS compared to normal human sinonasal tissue.[16] A recent study identified approximately 10-fold less AQP5 mRNA in CRSwNP specimens, [17] but the low patient numbers in their study may be a factor for this discrepancy.

The distribution of AQP protein in mucosa affected by CRS was similar to control specimens for most of the AQPs studied. However, greater expression of AQP4 and AQP5 proteins was detected at specific tissue sub-sites. More intense localization of AQP5 to the cytoplasm of glandular epithelial cells in CRSwNP, surface epithelial cells and AQP4 to the cytoplasm of glandular epithelial cells of CRSsNP was observed. This contrasts with the study by Shikani et al where less AQP5 protein was observed in CRSwNP specimens compared to CRSsNP or control.[17] However, analysis in that study only addressed intensity of labeling in the entirety of the surface epithelium, whereas we provided more detailed assessment of cellular localization patterns. Analysis of AQP1 protein localization failed to reach significance in this study ($p=0.065$), but another study identified increased AQP1 protein expression in polyp fibroblasts and connective tissue.[13] Finally, results of this study are consistent with several Chinese studies which also found increased AQP1and AQP5 proteins in surface and glandular epithelium of CRSwNP.[15, 33-36] However, direct comparison with these studies should be interpreted with caution due to phenotypical differences in CRS affecting differing ethnic groups (i.e. eosinophilic vs. neutrophillic inflammation in CRS and nasal polyps). [37, 38]

Cytoplasmic AQP localization

The significance of our finding of increased intracellular (cytoplasmic) AQPs in CRS is currently unclear. Some intracellular AQPs have been identified to date including in the renal tract, AQP2 being trafficked from intracellular vesicles to cell membrane in response to vasopressin,[39] while AQP6 function inside the cell is less clear.[40] Furthermore, in cerebral edema it was observed that AQP4 failed to insert at the blood-brain-barrier, instead localizing within the cell, and resulted in water retention in the brain. [41][42] While AQPs are known to localize to the cell membrane, work by other investigators has confirmed that AQPs are more than 'just' water channels with ongoing investigation of involvement in other cellular processes such as cell cycle regulation and tissue remodeling via cell volume regulation, glycerol transport, cell migration and apoptosis.[43, 44]

Limitations and future work

Although unlikely, it is possible that the small but statistically significant decrease in AQP4 and AQP11 mRNA expression may be due to a type 1 error given the low expression level of these AQPs. The lack of difference in mRNA expression between control and CRS disease may reflect the use of whole thickness sinonasal specimens in the qRT-PCR studies and more specific mRNA expression information may be obtained with future studies focusing on micro- dissection of the surface epithelium or sub-mucosal glandular epithelium. Further studies are required to investigate AQP function and regulation in normal sinonasal mucosa, better characterize water flux across sinonasal tissue, and investigate the potential role AQPs may play in tissue remodeling and polyp formation.

Conclusion

In summary, this is the most comprehensive study to date of AQP expression in normal and CRS sinonasal tissues. Differences in AQP expression profiles between normal and CRS diseased tissues were observed at the mRNA and protein levels. We hypothesize that altered AQP expression and/or cellular localization in CRS disease could disrupt water flow and contribute to thickened secretions, edema and/or nasal polyp formation.

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Table 1

Aquaporin	Antibody info	Dilution	Company	Antigen Retrieval
AQP1	Rabbit anti-human	1:12000	Alpha diagnostics	No retrieval required
AQP3	Rabbit anti-human, polyclonal	1:5000	Millipore diagnostics	Citric acid
AQP4	Mouse, monoclonal	1:500	AbD Serotec	Trypsin
AQP5	Rabbit, monoclonal	1:1000	Epitomics	Alkaline
AQP7	Rabbit anti-rat/human	1:100	Alpha diagnostics	Alkaline
AQP11	Rabbit anti-mouse	1:200	Alpha diagnostics	Trypsin

Table 1: Primary anti-aquaporin antibodies and conditions for immunohistochemical localization of AQPs

Table 2

AQP	Disease Group	Surface Epithelium Apical	Surface Epithelium Basolateral	Surface Epithelium Cytoplasm	Gland Epithelium Apical	Gland Epithelium Basolateral	Gland Epithelium Cytoplasm	Vasculature	Connective Tissue
AQP1	Normal	-	-	-	-	-	-	++ (+/- to ++)	+ (- to ++)
	CRSwNP	-	-	-	-	-	-	++ (+/- to ++)	++ (+ to +++)
	CRSsNP	-	-	-	-	-	-	++ (+ to ++)	++ (+ to +++)
AQP3	Normal	+ (- to ++)	+++ (- to +++)	++ (- to +)	+/- (- to +)	++ (- to +++)	- (- to ++)	-	-
	CRSwNP	+ (- to ++)	++ (- to +++)	+ (- to ++)	+/- (- to +)	++ (+ to +++)	+ (+/- to +)	-	-
	CRSsNP	+ (- to ++)	+++ (- to +++)	+ (- to ++)	+/- (- to +)	+++ (+ to +++)	+ (+/- to +)	-	- (- to +/-)
AQP4	Normal	+/- (- to +)	- (- to +)	+/- (- to +)	- (- to +/-)	- (- to +/-)	- (- to ++)	-	+ (+/- to +)
	CRSwNP	- (- to +)	-	- (- to ++)	- (- to +/-)	- (- to +/-)	+ (- to ++)	-	+ (+/- to ++)
	CRSsNP	+ (- to ++)	+ (- to ++)	+ (- to ++)	- (- to +++)	+/- (- to ++)	**++ (- to ++)**	-	+ (- to ++)
AQP5	Normal	++ (- to +++)	++ (- to +++)	+ (- to +)	+++ (- to +++)	+ (- to +)	+ (- to ++)	-	-
	CRSwNP	+ (+ to +++)	++ (++ to +++)	+ (+ to ++)	+++	++ (+ to ++)	**++ (+ to ++)**	-	-
	CRSsNP	+++	+++	**++**	+++	++ (++ to +++)	+ (+ to ++)	-	-
AQP7	Normal	-	-	-	-	-	- (- to ++)	-	-
	CRSwNP	-	-	+ (+/- to +)	-	-	+/- (- to +)	-	-
	CRSsNP	-	-	+ (- to +)	-	-	+/- (- to +)	-	-
AQP11	Normal	-	-	- (- to +/-)	-	-	- (- to +/-)	-	- (- to +/-)
	CRSwNP	-	-	-	-	-	-	-	+/- (- to +/-)
	CRSsNP	-	-	- (- to +/-)	-	-	- (- to +/-)	-	+/- (- to +/-)

Table 2: Immunohistochemistry localization intensity scores by location

All tissue sections were scored for intensity of immunohistochemical AQP labeling at the anatomical locations listed in the table using a Likert scale: -, +/-, +, ++, +++, or "not present" when the anatomical structure wasn't present in the section. Data presented as median (entire range). Absence of range indicates all scores were the same. Boxes shaded grey indicate localization was significantly different to control (p<0.05 Kruskall Wallis with Dunn's correction for multiple comparisons).

Table 3

AQP	Relative mRNA concentration			Kruskal-Wallis	Dunn's post-hoc test	
	Control Median (IQR)	CRSwNP Median (IQR)	CRSsNP Median (IQR)	p value	Control vs CRSwNP	Control vs CRSsNP
AQP0	0.00108 (0.0005-0.0105)	0.000886 (0.0003-0.0014)	0.000955 (0.0003-0.0018)	0.6819	N/A	N/A
AQP1	3.59 (2.03-10.56)	4.73 (2.67-7.18)	3.32 (1.89-5.42)	0.4737	N/A	N/A
AQP2	0.0165 (0.001-0.0237)	0.0165 (0.0115-0.0233)	0.0167 (0.0062-0.0203)	0.9488	N/A	N/A
AQP3	1.55 (1.01-4.92)	5.09 (3.42-9.82)	2.54 (1.51-6.75)	0.0519	N/A	N/A
AQP4	0.12 (0.106-0.200)	0.0692 (0.055-0.092)	0.0846 (0.070-0.155)	0.0061	Significant 1.7x down	N/A
AQP5	2.49 (0.797-7.13)	2.1 (1.26-3.25)	3.14 (1.17-5.46)	0.5096	N/A	N/A
AQP6	0.0292 (0.0205-0.0397)	0.0265 (0.0219-0.0339)	0.0296 (0.0169-0.0389)	0.8889	N/A	N/A
AQP7	0.028 (0.0232-0.0393)	0.0175 (0.0140-0.0269)	0.0286 (0.0191-0.0403)	0.0680	N/A	N/A
AQP8	0.00109 (0.000754-0.00446)	0.0018 (0.00108-0.00341)	0.00172 (0.000857-00238)	0.7313	N/A	N/A
AQP9	0.0231 (0.0160-0.0569)	0.0394 (0.0295-0.0625)	0.0299 (0.0217-0.142)	0.5171	N/A	N/A
AQP10	0.00683 (0.00550-0.00833)	0.00286 (0.00236-0.00669)	0.006799 (0.00285-0.0136)	0.0866	N/A	N/A
AQP11	0.0935 (0.0789-0.155)	0.0769 (0.0579-0.0877)	0.0943 (0.0902-0.111)	0.0129	Significant 1.2x down	N/A

Table 3: Relative mRNA concentrations for AQP0-11

Relative concentration of mRNA for each AQP is presented as the median (with interquartile range). Data analyzed using Kruskal-Wallis with Dunn's correction for multiple comparisons. Boxes shaded grey indicate mRNA expression was statistically significantly different compared to control (p <0.05 Kruskall Wallis with Dunn's correction for multiple comparisons). N/A indicates a site where statistical significance was not reached with KW testing, therefore Dunn's was not applied.

Figure 1

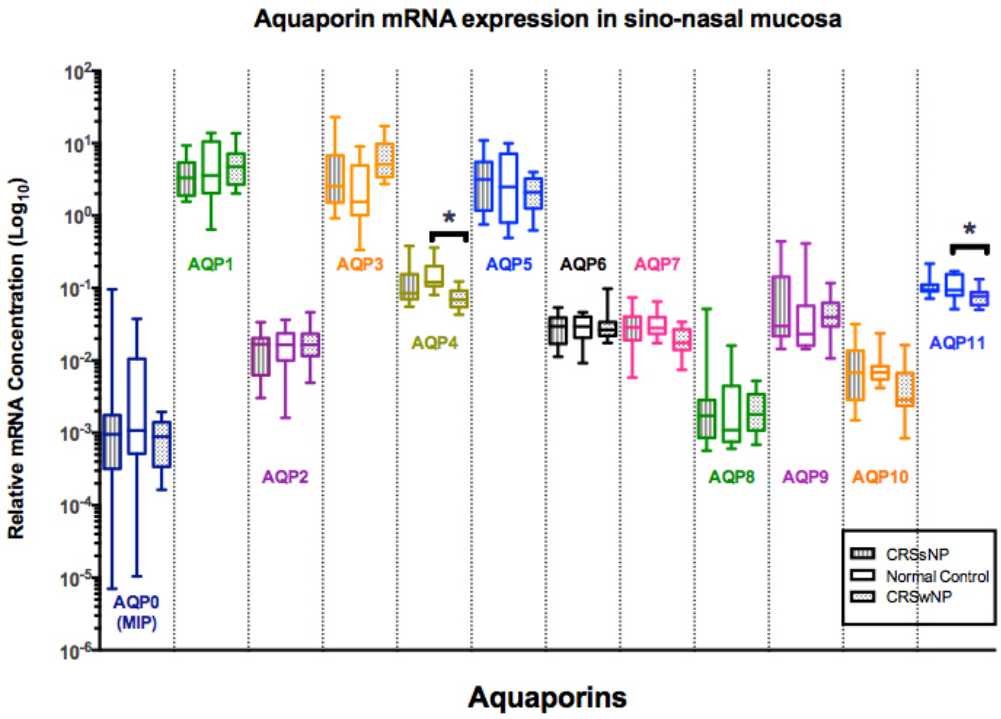

Figure 1: Relative concentration of AQP0-AQP11 mRNA in sinonasal mucosa

*Data presented as boxplot with median, interquartile range, and range. A logarithmic scale is used due to the wide range of concentrations. The relative concentration of mRNA for several aquaporins was extremely low. No AQP12b mRNA was identified and the AQP has been omitted from the graph. * = p <0.05 Kruskal Wallis with Dunn's correction for multiple comparisons.*

Figure 2

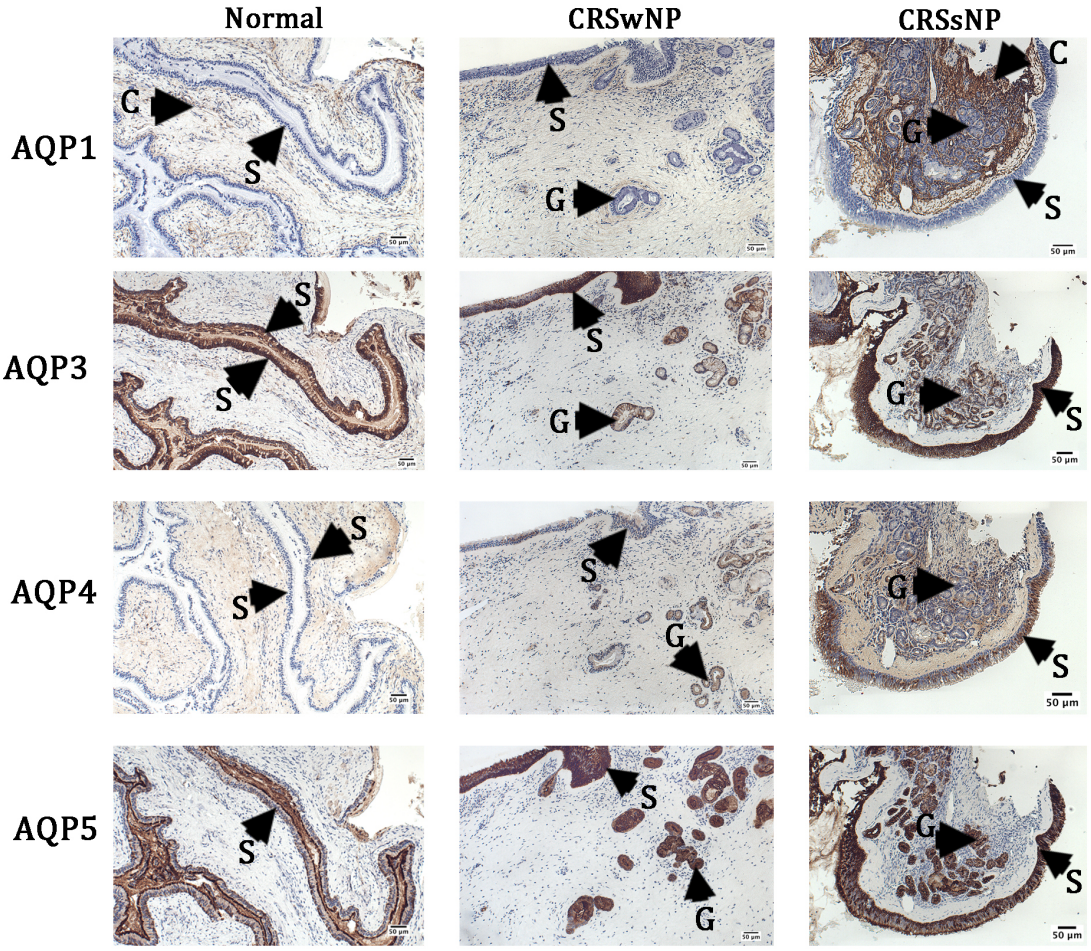

Figure 2: Immunohistochemical localization of AQP1, AQP3, AQP4 and AQP5

These photomicrographs demonstrate distribution of immunohistochemical localization of AQPs within normal sinonasal tissue, CRSwNP, CRSsNP.

***Normal**: The area of tissue photographed contains well-preserved surface epithelium, with minimal glandular epithelium.*

***CRSwNP:** The CRSwNP tissue photographed (not polyp) demonstrates thickened basement membrane under the surface epithelium, increased connective tissue cellularity and glandular hypertrophy.*

***CRSsNP:** The CRSsNP tissue demonstrates thickening of the basement membrane, surface epithelial hypertrophy, mucus gland hyperplasia and more fibrosis in the extracellular matrix.*

Figure 3

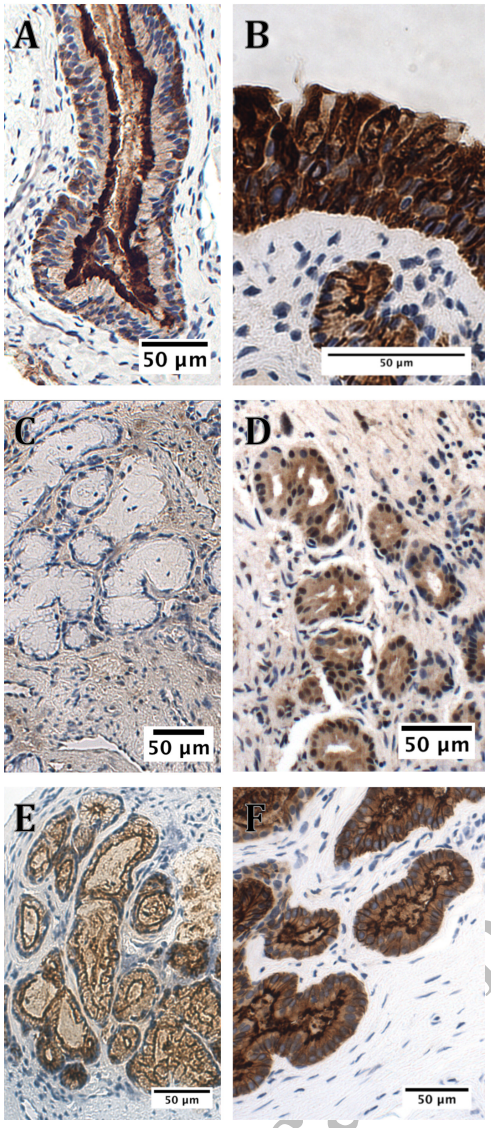

Figure 3: Immunohistochemical labeling – Study results

Photomicrographs demonstrating the three anatomical sub-sites where differences in AQP localization were observed between normal and CRS sinonasal tissues.

(A) normal: Apical AQP5 localization in surface epithelium, (B) CRSsNP: predominantly cytoplasmic AQP5 localization in surface epithelium , (C) normal: minimal AQP4 in glandular epithelium, (D) CRSsNP: greater cytoplasmic AQP4 expression in glandular epithelium, (E) normal: Apical AQP5 localization in glandular epithelium, (F) CRSwNP: predominantly cytoplasmic AQP5 localization in glandular epithelium.

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