Chronic Rhinosinusitis with Nasal Polyps

Symptoms, Heredity and Genetics

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Cover illustration: Manhattan plot from the DFAM analysis in the paper "A family-based genome-wide association study of chronic rhinosinusitis with nasal polyps implicates several genes in the disease pathogenesis." https://doi.org/10.1371/journal.pone.0185244.g002 by Anton Bohman used under CC BY4.0 / Labels removed

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In loving memory of my father Lars Bohman

To my family

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ABSTRACT

Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by long-term inflammation of the paranasal sinuses combined with bilateral glassy protuberances from the middle meatus of the nasal cavity. This disease has an unknown cause, affects approximately 3% of the population and causes symptoms from the upper airways. This thesis addresses the heredity, symptoms and possible genetic factors of chronic rhinosinusitis with nasal polyps.

METHODS/RESULTS: **Paper I** investigates the prevalence of nasal polyps in a group of 410 first-degree relatives to patients with the same condition using nasal endoscopy and compares them to a control group of 1387 individuals from a previous study. 13.4% of the relatives had nasal polyps themselves, compared to 2.7% in the control group. The relative risk of the first-degree relatives having nasal polyps when compared to the control group was 4.9.

Paper II studies the symptoms and risk factors of 367 patients with CRSwNP and compares them to 1349 polyp-free controls. Symptoms and risk factors were gathered by a structured interview and compared in a multiple logistic regression model. Higher age, male sex, nasal blockage, impaired sense of smell, nasal secretions and asthma was more common among subjects with CRSwNP whereas smoking was less frequent.

Paper III is a family-based genome-wide association study that compares single nucleotide polymorphisms between 406 participants with CRSwNP and 376 of their polyp-free first-degree relatives. After association testing and post-GWAS analysis; *HLCS*, *HLA-DRA*, *BICD2*, *VSIR* and *SLC5A1* were the most significant. Of these five genes, only *HLA-DRA* had been implicated in CRSwNP previously.

Paper IV measures the expression levels of ten of the most significant genes from Paper III in peripheral blood from 76 individuals with CRSwNP and 45 of their polyp-free relatives and studies their eQTL patterns. *NDUFS5*, *CPEB3*, *HLCS* and *BICD2* were upregulated in cases. *HLCS*, *LYZ*, *PDGFD* and *TIAM1* showed differences in expression when examining participants with different genotypes.

CONCLUSIONS: First-degree relatives of patients with CRSwNP have an almost fivefold increased relative risk of having nasal polyps themselves when compared to controls. Nasal secretion, nasal blockage and decreased sense of smell are more common among subjects with CRSwNP than among controls. *HLCS*, *BICD2*, *VSIR* and *SLC5A1* are potential new genes of interest in CRSwNP. *HLA-DRA* is strengthened as a research target. *NDUFS5*, *CPEB3*, *HLCS* and *BICD2* are upregulated in peripheral blood samples from patients with CRSwNP when compared to controls. *HLCS*, *LYZ, PDGFD* and *TIAM1* displayed differences when comparing allelic expression.

Keywords: Nasal Polyps, Genetics, Signs and Symptoms, Genome-Wide Association Study, Gene Expression

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SAMMANFATTNING PÅ SVENSKA

Näspolyper är en kronisk sjukdom, som kännetecknas av blod- och cellfattiga, ofta transparanta, utväxter i näsan. Polyperna anses bero på kronisk inflammation i näs- och bihåleslemhinnan och man använder därför ofta beteckningen kronisk rhinosinuit med näspolyper. Knappt 3 % av Sveriges befolkning lider av näspolyper, vilka kan ge stora problem med andning, nästäppa, snuva och nedsatt luktsinne. Små polyper behöver dock inte ge symtom.

Orsaken till näspolyper är okänd men det finns en koppling till astma och cystisk fibros, som bägge är allvarliga sjukdomar. Man har misstänkt att det finns ett ärftligt inslag för näspolyper, men det har inte bevisats. Genetiska faktorer har studerats tidigare, men mycket är fortfarande okänt om gener kan påverka utvecklingen av sjukdomen. Syftet med detta forskningsprojekt var att utforska symptomen, ärftligheten och genetiska aspekter på näspolyper.

I **Delarbete 1** undersöktes 410 nära släktingar (föräldrar, barn och syskon) till patienter med näspolyper med endoskopi av näsan. De jämfördes med en slumpvis uttagen kontrollgrupp av 1387 vuxna skaraborgare, vilka undersökts i en tidigare studie. Risken för näspolyper var nästan fem gånger större hos släktingarna till patienter med näspolyper jämfört med kontrollmaterialet. Näspolyper var vanligare hos manliga släktingar jämfört med kvinnliga och även vanligare med stigande ålder.

I **Delarbete 2** jämfördes förekomsten av dagliga symtom, riskfaktorer och rökvanor hos 367 polyppatienter med 1349 slumpvis utvalda vuxna personer utan näspolyper med hjälp av en strukturerad intervju. Manligt kön, hög ålder och astma var vanligare hos försökspersoner med näspolyper medan rökning var mindre vanligt. Patienter med näspolyper hade oftare dagliga besvär med nästäppa, snuva och nedsatt luktsinne jämfört med friska kontroller.

Delarbete 3 är en familjebaserad genomvid associationsstudie, en studie där förekomsten av små skillnader spridda över hela arvsmassan jämförs mellan sjuka och friska individer. Studien använde en kombination av genetisk associationstestning och nedärvning bland släktingar för att undersöka om det fanns genetiska skillnader mellan 406 individer med näspolyper och 376 av deras friska släktingar. Efter analys av de 1000 markörer som uppvisade störst skillnad mellan sjuka och friska och 138 gener där dessa markörer överlappar, jämfördes resultaten med en databas över genuttryck. Även en analys av genetiska skillnader som påverkar genuttryck utfördes. Genen *HLA-DRA* som tidigare misstänkts ligga bakom näspolyper var signifikant efter samtliga analyser liksom fyra gener som inte varit aktuella i sjukdomsutvecklingen tidigare; *HLCS*, *VSIR*, *BICD2* samt *SLC5A1*.

I **Delarbete 4** gjordes ytterligare analyser av 10 av de riskgener som identifierades i delarbete 3. Uttrycket av dessa riskgener jämfördes med uttrycket av en referensgen både för 76 försökspersoner med näspolyper och också 45 av deras friska släktingar. På liknande sätt studerades hur genetiska skillnader påverkade genuttrycket hos försökspersonerna. Generna *HLCS*, *BICD2*, *TIAM1*, *PDGFD*, *CPEB3*, *NDUFS5* och *LYZ* var mest signifikanta.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Bohman A, Oscarsson M, Holmberg K, Johansson L, Millqvist E, Nasic S, Torinsson-Naluai Å, Bende M.**
Heredity of nasal polyps.
Rhinology. 2015 Mar;53(1):25-8.

II. **Bohman A, Oscarsson M, Holmberg K, Johansson L, Millqvist E, Nasic S, Bende M.**
Relative frequencies of symptoms and risk factors among patients with chronic rhinosinusitis with nasal polyps using a case-control study.
Acta Otolaryngol. 2018 Jan;138(1):46-49.
This is the authors accepted manuscript of an article published as the version of record in 2018©
Taylor & Francis (Acta Oto-Laryngologica AB Ltd)
https://doi.org/10.1080/00016489.2017.1366052

III. **Bohman A, Juodakis J, Oscarsson M, Bacelis J, Bende M, Torinsson-Naluai Å.**
A family-based genome-wide association study of chronic rhinosinusitis with nasal polyps implicates several genes in the disease pathogenesis.
PLoS One. 2017 Dec 18;12(12):e0185244.

IV. **Bohman A, Oscarsson M, Annor G, Bende M, Torinsson-Naluai Å.**
A study of expression of genes implicated in a genome-wide association study on chronic rhinosinusitis with nasal polyps.
(Manuscript)

CONTENT

ABBREVIATIONS

cDNA	Complementary DNA
CF	Cystic fibrosis
CI	Confidence interval
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CT	Threshold cycle
ΔΔCT	Delta-Delta CT
EMMAX	Efficient mixed model association expedited
EPOS 2012	European position paper on rhinosinusitis and nasal polyps, 2012 edition
eQTL	Expression quantitative trait loci
FESS	Functional endoscopic sinus surgery
GEO	Gene expression omnibus
GO	Gene ontology
GWAS	Genome-wide association study
ICD10	International Classification of Diseases
INCS	Intranasal corticosteroids
KEGG	Kyoto encyclopedia of genes and genomes
LD	Linkage disequilibrium
mRNA	Messenger ribonucleic acid
MuTHER	Multiple tissue human expression resource
NP	Nasal polyps
OR	Odds ratio
qPCR	Quantitative polymerase chain reaction
RR	Relative risk
SGLT1	Solute carrier family 5 (sodium/glucose cotransporter) member 1
SNP	Single-nucleotide polymorphism
TDT	Transmission disequilibrium test

1 INTRODUCTION

Patients with nasal polyps are common in primary healthcare and otorhinolaryngological departments world-wide. This disease leads to suffering, decreased quality of life and absenteeism from work. Patients with nasal polyps are commonly treated with a combination of medical and surgical methods but the polyps and their associated symptoms often recur after a period of time. Despite how common these patients are and their life-long suffering, little is known about the pathogenesis of the disease.

To increase the knowledge of nasal polyps this project utilises studies on the heredity, symptomatology and possible genetic mechanisms behind the disease.

1.1 NASAL POLYPS

Nasal polyps (NP) are most often described as glassy protuberances from the middle meatus of the nasal cavity. Both the macroscopic and histological appearance can vary between individuals depending on factors such as the presence or absence of tissue eosinophilia [1]. This thesis focuses on idiopathic nasal polyps and not masses in the nasal cavity that may have a similar macroscopic appearance such as antrochoanal polyps, benign or malignant tumours.

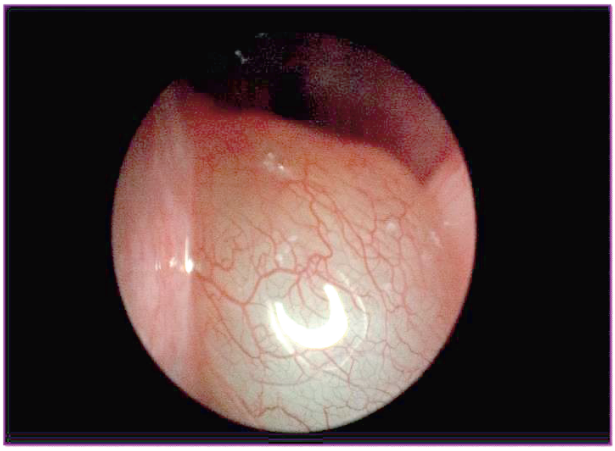

Figure 1. Nasal polyp, endoscopic view

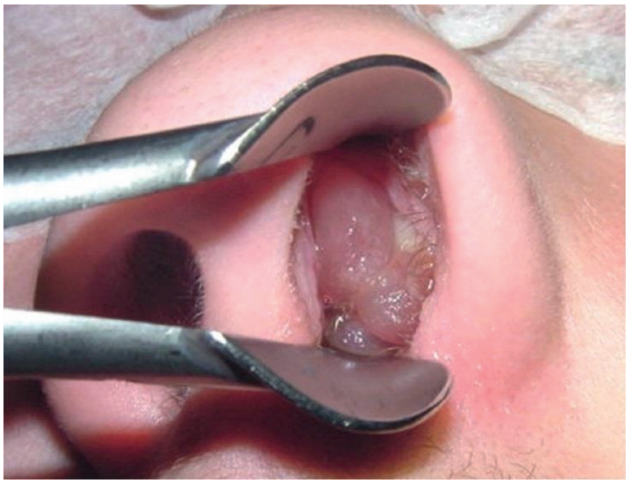

Figure 2. Extensive nasal polyps in the left nasal cavity, clearly visible using a nasal speculum

1.2 CLASSIFICATION OF NASAL POLYPS AND CHRONIC RHINOSINUSITIS

One of the most used classifications of nasal polyps and inflammation of the nasal cavity and paranasal sinuses is the European position paper on rhinosinusitis and nasal polyps (EPOS 2012). This position paper defines chronic rhinosinusitis (CRS) as:

Presence of two or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip):

-- ± facial pain/pressure;

-- ± reduction or loss of smell;

For ≥12 weeks; with validation by telephone or interview [2].

Furthermore, CRS is divided into two sub-groups: chronic rhinosinusitis without nasal polyps (CRSsNP) and chronic rhinosinusitis with nasal polyps (CRSwNP). CRSwNP is defined as CRS (using the symptoms above) and the presence of bilateral, endoscopically visualized polyps in the middle meatus.

In turn, CRSsNP is defined as CRS and the absence of visible nasal polyps in the middle meatus, if necessary following decongestant [2].

Even though EPOS 2012 and its division of CRS into two subtypes with or without NP is a commonly used classification, later studies have suggested that it may be advantageous to subdivide these conditions further using other criteria due to differences in histopathology and/or inflammatory pathways, sometimes attributed to regional disparities [3, 4].

1.3 EPIDEMIOLOGY

The prevalence of CRSwNP has been investigated using different methods, two surveys based on questionnaires [5, 6] found the prevalence to be 4.3% and 2.2% respectively and the largest population-based investigation using nasal endoscopy to date found a prevalence of 2.7% [7]. Increasing age is a risk factor [6, 7, 8, 9], as well as male sex [7, 8, 10, 11, 12].

1.4 RELATION TO OTHER CONDITIONS

1.4.1 ALLERGY

For CRS in general there is data to support the idea that allergy is linked to the disease [13]. However, this relationship is not established in CRSwNP, the prevalence of NP among subjects with allergic rhinitis [9] is comparable to the prevalence among the general population [5, 6, 7] and atopy is not more common among NP patients than it is among controls [14, 15, 16].

1.4.2 ASTHMA

CRSwNP is highly associated with asthma. Patients with asthma have been reported to be more likely to suffer from CRSwNP than subjects without asthma [5, 7, 9]. Similarly, studies show a significantly higher prevalence of asthmatics in study groups with CRSwNP when compared with controls [17, 18].

1.4.3 ASPIRIN SENSITIVITY

Patients with aspirin sensitivity have a prevalence of CRSwNP between 36-71% [14, 19, 20, 21]. When the combination of aspirin sensitivity, asthma and NP occurs, the condition is referred to as the ASA triad or Samter's triad [22].

1.5 SYMPTOMS

A study of 1784 patients with CRSwNP found that the most commonly reported symptoms were nasal blockage (97%), altered sense of taste and/or smell (90%) and the need to blow one's nose (80%) [23]. A different study investigated symptoms in 165 patients with NP; the most common symptoms were nasal blockage (88%), anosmia (78%) and rhinorrhoea (66%) [17]. However, sinonasal symptoms are frequent among the general population [24] and prior to this thesis that was no study that compared the symptoms of subjects with CRSwNP with those of a control group drawn at random from the general population.

1.6 MEDICAL TREATMENT

In the modern era, intranasal corticosteroids (INCS) constitute the main therapy for CRSwNP alongside surgery and systemic steroids. Many studies have investigated the effect of INCS on either symptoms, polyp size or nasal airflow but the meta-analysis in EPOS 2012 could only pool a handful of studies due to different study designs [2]. Pooled data from seven studies showed a significantly better result on symptom scores for the treatment group compared to the placebo group [25, 26, 27, 28, 29, 30, 31]. When combined, three studies could show a significantly lower polyp score among the group treated with INCS compared to a placebo group [27, 32, 33]. The study group also had a significantly better peak nasal inspiratory flow when data from seven studies [26, 27, 29, 31, 34, 35, 36] were analysed together.

Systemic steroids have been used to treat CRSwNP for decades and several studies have been able to show a positive effect on both patient reported outcomes and endoscopic results when compared to placebo [37, 38, 39].

Other suggested medical treatment modalities for CRSwNP include antibiotics (long-term or short-term), anti-IgE, anti-IL5, antihistamines, furosemide, aspirin desensitization, immunosuppressants, antimycotics, capsaicin and leukotriene antagonists but there is not enough evidence to support these treatments according to EPOS 2012 [2].

1.7 SURGICAL TREATMENT

Functional endoscopic sinus surgery (FESS) was introduced in the twentieth century and is the dominating surgical method for the treatment of CRSwNP today. The procedure is performed endoscopically and usually as outpatient

surgery. The extent of the surgery varies, but it typically involves removing the uncinate process of the ethmoid bone, establishing a middle meatal antrostomy to the maxillary sinus and a partial or complete ethmoidectomy. More extensive procedures can include complete bilateral sphenoethmoidectomies and surgery of the frontal recess and sinuses. The goal was originally focused around the concept of improving the ventilation and drainage of the paranasal sinuses [40] but there are researchers who advocate that improving delivery of topical medical treatment is also of importance [41]. Even though there are possible major complications such as cerebrospinal fluid leakage or orbital complications, studies have shown that FESS is an efficient and safe method for addressing CRSwNP [42]. Despite this, the polyps often recur and require revision surgery [43, 44]. The optimal extent of endoscopic sinus surgery is under debate but there is data that suggests more extensive surgery might be beneficial, at least with regard to the distribution of topical medication [45]. Other researchers have proposed an even more radical approach that includes the endoscopic removal of all affected mucosa down to the periosteum (the "reboot procedure") for use in select patients with type 2 inflammatory response [46]. In spite of this, less invasive procedures such as simple polypectomies are still used in some cases.

1.8 PATHOGENESIS AND AETIOLOGY

One of the first modern attempts to explore the aetiology of CRS was the "fungal hypothesis". This theory connected the cause of all CRS to a misguided host response to Alternaria fungi [47, 48]. Most researchers have subsequently opposed this theory as an explanation to CRS in general [49] but there is still support for the role of fungi as disease modifiers in some forms of CRS [50].

Another theory is the "staphylococcal superantigen hypothesis", this theory describes the formation of nasal polyps as a result of bacterial exotoxins and their effect on cells in the nasal mucosa and the immune system [51, 52]. This effect can be found in roughly half of patients with CRSwNP and staphylococci are therefore mostly viewed as another example of a disease modifier rather than an actual cause [53].

A related theory, the "biofilm hypothesis" suggests that the formation of bacterial biofilms in the sinuses and nasal cavity is a possible important factor in the development of CRS. As of yet there is no absolute data on biofilm as a cause of CRS [54].

As opposed to the above mentioned theories based on microorganisms, there are at least two theories that focus on host factors rather than an outside

stimulus: The “eicosanoid hypothesis” and the “immune barrier hypothesis”. The eicosanoid hypothesis attributes defects to the eicosanoid pathway not only to aspirin intolerance but also to CRS in general [53, 55]. However, the data is still rather limited and the modest clinical efficacy of leukotriene pathway inhibitors as a treatment for CRSwNP suggests that this pathway may not be a major factor in the pathogenesis of CRS [2].

The immune barrier hypothesis suggests that defects in the innate immune system and mechanical barrier predisposes to the pathogenesis of CRS when exposed to relatively common microorganisms. One of the first pieces of evidence to support this theory came from patients with cystic fibrosis (CF). These patients have an impaired mucociliary flow and a very high incidence of CRS (both CRSwNP and CRSsNP) [56]. There is also evidence that mutations in the gene that causes CF, *CFTR*, can lead to CRSwNP without any other clinical manifestations of CF [57]. Furthermore, there are studies that point to impaired mucociliary clearance as being present more broadly in CRS [58, 59]. Other investigators have also reported a defective mechanical barrier in patients with CRSwNP [60, 61].

In summary, there is support for a role of both host and environmental factors but the aetiology and pathogenesis of CRSwNP remains unclear and an important field of research.

1.9 HEREDITY

Earlier studies have shown that a family history of NP is more frequent among patients with NP than in controls [17, 62, 63]. However these figures are based on questionnaires and interviews, there was no study on familial aggregation of NP were the presence of the condition was based on endoscopic investigation (which is mandatory for diagnosis) prior to this thesis.

1.10 GENETIC STUDY METHODS

Disease genetics can be investigated in different ways, using for example linkage studies, candidate gene studies, genome-wide association studies (GWAS), and studies of gene expression or expression quantitative trait loci (eQTL).

A linkage study uses related individuals to explore the relationship of two genetic loci (or a locus and a trait or two traits) using the tendency of DNA sequences in close proximity to be transmitted from parent to offspring

together. The higher the association between a trait and a loci, the more likely it is for that loci to contain genetic variants which influence the trait in question. Genetic linkage is one of several factors that influences linkage disequilibrium (LD) i.e. the non-random association of alleles at different loci. If alleles at different loci occur together more often or seldom than expected by random chance, they are said to be in LD. Another potential cause of LD in a population is so called population stratification which is a systematic difference in allele frequencies between different groups (subpopulations) within the studied population, e.g. caused by different genetic backgrounds.

A candidate gene study tests the association between a single-nucleotide polymorphism (SNP) or other types of genetic variations and a trait (such as CRSwNP). This association can either be direct i.e. the SNP itself influences the trait, or indirect i.e. the SNP is in LD with a genetic variant that influences the trait. As the name implies candidate gene studies rely on a previously formulated hypothesis that the gene influences the trait you are investigating in some manner.

In contrast, a GWAS tests associations between a trait and large number of SNPs (usually hundreds of thousands or millions) spread over the entire genome and is therefore not reliant on a previously formulated hypothesis. Instead, a GWAS is hypothesis-free and hypothesis-generating. The results from a GWAS are ideally followed up by a more focused study on the implicated genes in a different study population. GWAS have been successful in detecting genetic variants associated with many common diseases [64] but most of the estimated heritability is still unaccounted for, this is often referred to as "missing heritability" [65]. Due to the large amount of statistical tests performed in these studies there is a high risk of type I errors due to multiple testing, therefore strict levels of significance are commonly applied with a p-value below $5x10^{-8}$ being one of the more accepted [66]. However, this does not mean that all signals above that threshold are automatically false and do not contribute to the investigated trait. Some authors have proposed that these strict significance levels could increase the risk of missing common variants that have an individual effect that is too small to pass a threshold of $p<5x10^{-8}$ but due to there being so many of these common variants, their additive and epistatic effects could possibly explain at least some of this "missing heritability" [67]. Another idea regarding the cause of GWAS inability to explain most of the heritability of many traits is that a GWAS is typically best suited for finding common variants because most microchip arrays use SNPs with relatively high minor allele frequencies. This can cause rare variants to not be detected simply by not being in complete LD with any of the genotyped SNPs in a specific microchip array. Rare variants that might have a large effect

on the trait or disease could therefore possibly go unnoticed, e.g. multiple GWAS on breast cancer have so far failed to detect *BRCA1* [64] which was discovered by linkage [68] and is one of the more predominant breast cancer susceptibility genes uncovered to date.

As these examples suggest, linkage studies, candidate gene studies and GWAS have different pros and cons. A linkage study is typically better suited to finding rare variants compared to a GWAS but due to the necessity of using a pedigree of some sort (i.e. family members need to be sampled), these study designs can be difficult and laborious to complete. Candidate gene studies do not require related individuals but instead require the investigators to have a preconception of which markers and genes could possibly affect the investigated trait. A GWAS is useful in detecting common variants with relatively large effects and do not require any prior knowledge of possible associations between markers or genes and the trait in question, however they require large study populations and are not always equipped to detect rare variants or multiple common variants whose individual effects are very small.

Linkage or genetic association can be used to find genes of interest with regards to the pathogenesis of diseases with a complex inheritance pattern but cannot explain if or how these genes affect the condition. To test whether or not a certain gene or SNP is more or less likely to have some sort of functional importance with regards to the trait in question, other methods such as e.g. gene expression or eQTL can be applied.

When studying genetic expression, messenger ribonucleic acid (mRNA) levels are quantified using methods such as quantitative polymerase chain reaction (qPCR) or hybridization microarrays. qPCR can be used to measure either absolute or relative levels of nucleic acids. When using relative quantification, the expression of the gene in question is compared to that of a reference (housekeeping) gene chosen for its stability. This allows comparison between samples based on the fold-difference of gene product the qPCR produces of the target gene when compared to the reference gene. Results from different qPCR runs can also be tested simultaneously as factors such as variability in the quantity of RNA used can be corrected for using the expression of reference genes.

eQTL are variable regions in the genome, such as SNPs, that affect expression levels of mRNA. By combining expression profiling of selected tissue samples with microarray genotyping, gene expression patterns can be associated to SNP variations, creating a set of regulatory SNPs for each gene. An article comparing data from an eQTL database with GWAS data showed that SNPs

associated with complex traits were more likely to be eQTL than other SNPs matched for minor allele frequency from high density beadchips. The same article also suggested that annotating SNPs with information on eQTL patterns could aid in finding susceptibility loci for some diseases with a complex inheritance pattern [69]. Both genetic expression and eQTL can be used to follow up results from candidate gene studies, GWAS or linkage studies in an attempt to test the functional relevance of the results.

1.11 GENETIC STUDIES ON CRSwNP

The genetics of CRSwNP had prior to this thesis most often been investigated using association studies, most of these were studies on candidate genes but there was one pooling-based GWAS (i.e. a GWAS that uses genotyping of pooled samples instead of genotyping the samples individually) performed on patients with CRS (both CRSsNP and CRSwNP) [70]. This lead to a follow-up study on the gene *p73* where patients hetero- and homozygous for minor allele A in SNP rs3765731 were significantly less likely to have CRS [71].

Candidate gene studies published prior to this thesis had implicated numerous genes in CRSwNP including genes involved in arachidonic acid metabolism [72], tissue remodelling [73], immunity [73, 74, 75, 76] and inflammation [77, 78] as well as *CFTR* [79, 80].

Before this project there was only one study on genetic linkage and CRS performed on Hutterites, a religious isolate with a communal lifestyle that live in Canada and the western United States. In this study they found 8 subjects with CRS (the article does not specify whether any of them had CRSwNP) related to each other in a single 60 member pedigree and suggested a locus connected to CRS on chromosome 7 that included the locus for *CFTR*. However, after genotyping 38 mutations in the *CFTR* gene they were unable to detect variation that accounted for the linkage signal [81].

Prior to this thesis, there was no GWAS performed only on subjects with CRSwNP and no study exploring family-based data such as genetic linkage. Two years after Paper III was published, a GWAS using two large databases from Iceland and the United Kingdom found a loss-of-function variant in *ALOX15* to be protective against both NP and CRS in general [82]. The supplemental materials of this article state that International Classification of Diseases (ICD10) codes were used to decide which individuals were phenotype-positive or -negative in these databases (deCODE genetics and UK biobank respectively). However, there is no information on how many of these individuals had undergone nasal endoscopy (or CT scan in the case of

CRSsNP) and since the lack of an ICD10 J33.X-code does not exclude nasal polyps, there is no way of knowing how many of the controls were actually phenotype-positive and conversely, how many of the phenotype-positive subjects had a correct diagnosis.

1.12 THE GENETIC EXPRESSION AND eQTL PATTERNS OF CRSwNP

Studies where the expression of certain genes in nasal polyp tissue was compared to the expression in nasal mucosa from unaffected individuals have shown significantly altered expression levels in some instances. Examples of genes with altered expression levels from these studies are *CLCA1*, *CLCA2* and *CLCA3* [83], *PTGS2*, *POSTN*, and *IL4* [84], *IL19* [85], *MUC4* [86], *MMP1*, *MMP2* and *MMP9* [87], *OSM* [88] and *TIMP2* [89]. After measuring genome-wide mRNA levels in nasal polyp tissue and inflamed mucosa from 11 subjects with CRSwNP and concomitant asthma and comparing them to nasal mucosa from a control group of 17 individuals either free of sinonasal disease or with allergic rhinitis, a group of researchers found altered expression levels for 447 genes. *CCL18*, *CCL13*, *EMR3*, *IL1RL1* and *CRISP3* were among the genes with the largest transcription changes [90]. At the time of writing, Paper III and IV are the only studies that investigate eQTL in CRSwNP.

1.13 GENETICS OF ASTHMA

As mentioned above, CRSwNP is closely linked to asthma and the genetics of asthma have been investigated more thoroughly than those of CRS. Several GWAS have been performed including two studies with a total of more 20.000 participants, some of European origin as well as a large, more ethnically diverse group, residing in North America. The European consortium identified 10 genes (*IL1RL1*/*IL18*, *TSLP*, *IL33*, *SMAD3*, *HLA-DQ*, *ORMDL3*, *IL2RB*, *SLC22A5*, *IL13*, and *RORA*) [91], of which 6 could be confirmed by investigators in North America (*IL1RL1*/*IL18*, *TSLP*, *IL33*, *SMAD3*, *HLA-DQ* and *ORMDL3*) [92].

A later study found 16 SNPs in all 3 *ORMDL*s associated with asthma, 14 of these SNPs were found in *ORMDL3*. When comparing asthmatic patients to controls, the investigators detected a higher baseline expression of *ORMDL1* and *ORMDL2* in peripheral blood mononuclear cells from the asthmatic cohort [93].

AIMS

The over-arching goal of this thesis was to investigate important topics in CRSwNP, most significantly the heredity, symptomatology, relation to asthma as well as genetic association, linkage and gene expression.

Specific aims of the individual papers

Paper I

To explore the heredity of CRSwNP using endoscopy to examine the nasal cavities of first-degree relatives to patients CRSwNP and comparing this data with a control group drawn at random from the general population in a previous study

Paper II

To find answers about the relative frequency of symptoms, relationship with asthma and smoking habits in patients with CRSwNP by comparing this data with a population-based control group.

Paper III

To identify SNPs and genes associated with CRSwNP susceptibility using a family-based genome-wide approach.

Paper IV

To investigate the expression levels and eQTL of some of the proposed risk genes from Paper III in peripheral blood.

2 MATERIALS AND METHODS

2.1 PAPER I

A total of 410 first-degree relatives (parents, siblings or children) of 368 patients with nasal polyps were recruited for Paper I. All participants underwent nasal endoscopy to confirm or rule out the presence of nasal polyps in the middle meatus of the nasal cavity. These results were compared with results from a controlled randomized study where 1387 individuals drawn from the general population of the Swedish community of Skövde were investigated in the same manner. In the statistical analysis, comparisons of prevalence was done by either using chi-square tests or by calculating confidence intervals (CI) and relative risk (RR).

2.2 PAPER II

For Paper II, 367 patients with nasal polyps were compared to a control group consisting of 1349 participants without nasal polyps from the same previous study as the control group in Paper I. All individuals had been examined with nasal endoscopy to determine whether or not they had nasal polyps. Subjects without nasal polyps were chosen for the control group. The patients with nasal polyps and the controls underwent the same structured interview where information regarding medical history and symptoms from the upper and lower airways was gathered.

Nasal symptoms were identified using these questions: Are you bothered by nasal secretions?, ... nasal blockage? and ... sneezing?, respectively. If the answer was “yes”, the respondent was asked to indicate whether symptoms were experienced daily, frequently, or occasionally. Only symptoms that occurred daily were analysed in this study. Cough symptoms were identified using the question Do you have a cough?, followed by the same question regarding frequency [24, 94].

A question was asked concerning olfactory sensitivity, How do you rate your ability to detect weak odours?, used by Nordin et al. [95]. The answer alternatives were: normal, better than normal and worse than normal [96]. Only those who answered ”worse than normal” were classified with impaired sense of smell. Parosmia was identified by the question: Do you ever smell something, for example a rose or an orange, that should have a smell that you know, but instead, you smell a different odour, an off odour, a bad odour, or a burning odour? [97]. The reason for asking about parosmia in a time-wise

more general respect, rather than referring to a specific moment, was that parosmia is a condition that tends to fluctuate [98].

Individuals were considered to have asthma if they answered yes to any of the following questions: In the past 12 months, have you had symptoms of asthma or attacks of shortness of breath with wheezing? or Are you on asthma medication? [94, 95].

Sensitivity to cold air was assessed with the question: Do you have problems inhaling cold air? These questions could be answered by "yes" or "no" [94].

Smoking habits were identified by validated questions with high specificity [5]: Have you ever smoked regularly (i.e. almost every day at least for 1 year)?, and Do you currently smoke?.

In order to analyse both categorical and continuous variables, a multiple logistic regression model was used. Daily occurrence of nasal secretion, blockage, sneezing and cough were analysed. Impaired sense of smell was analysed to evaluate olfactory sensitivity. All symptoms in patients were compared with those in the control group and 95% confidence intervals (CIs) calculated.

A multiple logistic regression model was applied to the data to identify factors of prognostic significance for CRSwNP and odds ratios (ORs) calculated. A univariate analysis was used to aid in the selection of variables for the multivariate analysis. The following variables were selected in the model: age, sex, all respiratory symptoms, smoking (expressed as any-time and present smoking) and meaningful interactions between these variables. To keep this model as parsimonious and plausible as possible, stepwise selection (forward and backward) procedures were used. The significance level for inclusion and removal of a variable was set to 5%.

2.3 PAPER III

The same study group as in Paper II was used, 367 patients with nasal polyps, as well as 453 of their first-degree relatives (most of whom also featured as the study group in Paper I). Nasal polyps were identified with nasal endoscopy and all participants were subsequently classified as either phenotype-positive or phenotype-negative.

Peripheral blood samples were drawn from each individual and DNA was extracted from whole blood using an in-house protocol at KBiosciences (LGC

Genomics, Hoddesdon UK). The HiSeq Illumina platform was used for genotyping, 676 of the samples were run on Illumina Omni Express beadchips and the remaining 144 on the Illumina Core Exome array. Autosomal markers shared in both genotyping platforms were retained.

In order to generate SNP association values, two methods were used. DFAM (implemented in PLINK) combines the transmission disequilibrium test (TDT), the sibling TDT and an allelic test for unrelated cases and controls in a single Cochran-Mantel-Haenszel test for each marker [99]. Efficient Mixed Model Association eXpedited (EMMAX), implemented in Golden Helix SNP & Variation Suite v8.3.4 was also used. This method computes an empirical relatedness matrix of the samples, this relatedness is then used as a covariate in linear regression for each marker [100]. This test was performed using additive, dominant and recessive models and the smallest p-value from the three models was assigned to each SNP.

For both DFAM and EMMAX association results the top 1000 markers with the most significant association p-values were combined into intervals of SNPs in high LD (defined by pairwise $r2>0.25$).

Possible pathway enrichment within these regions was detected using INRICH software [101]. The significance of these overlaps was calculated by repeating the process 50.000 times with random genomic regions, matched in size and SNP density. INRICH analysis was performed separately using DFAM or EMMAX results and Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG)-based gene-sets. The 20 gene-sets with the highest enrichment p-values were retrieved from each of these setups. INRICH produced a list of genes which were located close to the top GWAS 'hits' in the genome and that share functional annotations. All genes retrieved in this way from the four INRICH analyses (DFAM+GO, EMMAX+GO, DFAM+KEGG, EMMAX+KEGG) were combined together, creating a list of target NP genes.

Publicly-available gene expression data, collected by Plager et al. [90], was retrieved from NCBI Gene Expression Omnibus (GEO) database ([102]; series accession number GSE23552). Two samples (aCRSm1 and aCRSm2) were excluded per authors' recommendation, leaving 20 case samples (all from patients with CRSwNP) and 17 control samples from either healthy individuals or patients with allergic rhinitis. Expression levels between the case and control groups were compared using the GEO2R interface. The differentially-expressed gene set is comprised of all genes corresponding to probes with significant difference in expression levels (Benjamini-Hochberg FDR <0.05).

For eQTL analysis, two datasets were used: Blood eQTL from Westra et al. [103] and Multiple Tissue Human Expression Resource (MuTHER) project [104]. These datasets consist of a list of regulatory SNPs for each gene produced by microarray genotyping, expression profiling of selected tissue samples and subsequent association of SNP variations with gene expression patterns.

In MuTHER project, the regulatory effects of each SNP were determined in adipose, skin tissues and lymphoblastoid cell lines (LCL). For each SNP-gene pair we have retained either LCL or skin data, corresponding to the tissue with more significant regulatory effect. We also excluded all SNPs with p-values >0.05 or absolute effect size (regression coefficient β) of <0.01. FDR of 0.5 was used as a cut-off for the Blood dataset, with no additional limits on effect size.

All eQTL SNPs for each gene of interest were extracted and classified according to the direction of their regulatory effect (up-regulating or down-regulating) to check for directed eQTL enrichment. The frequency of the allele bearing the reported regulatory effect was then determined in our GWAS cases and controls using PLINK [99]. The marker was then assigned to a bin depending on whether the regulatory allele shows higher frequency in cases or in controls. In this way, a 2x2 contingency table was constructed for each gene, where all SNPs fall into one of four quadrants (up-regulating + less frequent in cases; up-regulating + more frequent in cases; down-regulating + less frequent in cases; down-regulating + more frequent in cases). Fisher's test was used to test whether the regulatory effect and frequency difference are dependent.

To account for the effect of LD between SNPs, an iterative procedure was used to calculate the empirical significance. Genes were ordered according to the number of eQTL SNPs remaining after all filters; genes found in the differentially-expressed NP set (as described in the previous section) were removed; for each gene of interest with n SNPs, 500 genes with the same number n of SNPs are retrieved; if less than 500 genes have the required number of SNPs, genes with n+1 (then n+2, n+3…) SNPs are also retrieved, and n SNPs are randomly selected for analysis in those genes. Each gene is analysed in the same manner as the target gene. Resulting empirical distribution of p-values is used to determine the empirical significance for the gene of interest.

The workflow of the analysis in Paper III is summarized in Figure 3.

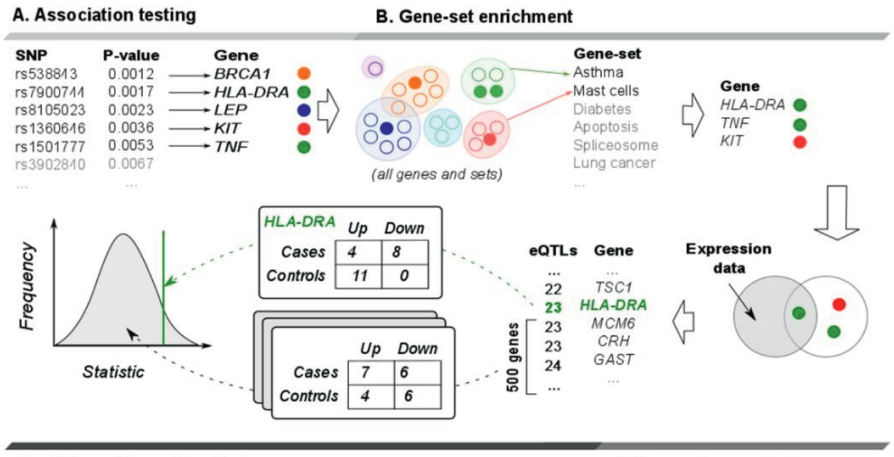

Figure 3. Workflow of the analysis in Paper III. A. Initially, SNP association p-values are produced by an association test. Based on these values, top 1000 SNPs are selected and annotated to nearby genes. B. INRICH software is then used to detect over-represented gene-sets (empty circles denote all genes that were not detected by GWAS). Genes from top 20 such sets are retrieved, and only the ones overlapping with GWAS hits are analysed further. C. Using publicly available expression data from NP samples, this gene list is filtered to retain only differentially expressed genes. D. For each of these remaining targets, known eQTL are assigned into bins based on effect direction (up- or down- regulating) and frequency distribution in our genotyping data (higher frequency in cases or in controls). Fisher's exact test is used to evaluate the observed distribution. 500 genes with equal or higher count of eQTL are analysed in the same way, and statistic values generated from this control set are then compared with the target gene statistic to estimate the empirical significance. Used in the paper "A family-based genome-wide association study of chronic rhinosinusitis with nasal polyps implicates several genes in the disease pathogenesis." https://doi.org/10.1371/journal.pone.0185244.g001 by Julius Juodakis and Anton Bohman used under CC BY4.0

2.4 PAPER IV

The study group for Paper IV consists of 76 patients with CRSwNP and 45 of their polyp-free relatives used as controls. Blood samples were taken from all participants and the total RNA was extracted. The Nanodrop 2000 spectrophotometer was used to assess the extracted RNA quantitatively and qualitatively. Complementary DNA (cDNA) was synthesized from the RNA samples using the Vilo kit.

Using the GeNorm algorithm [105] , a total of 23 reference (housekeeping) genes were tested for whole blood expression, *YWHAZ* had the highest stability value ($m<0.5$) and was chosen as a reference gene for normalization.

Quantitative gene expression analysis was performed using qPCR with TaqMan chemistry (Thermo Fisher Scientific Inc., CA, USA). To obtain a final reaction volume of 2 µl per gene and sample, a reaction mixture consisting of 1 ng/reaction cDNA together with Master Mix was added to all genes simultaneously using a Nanodrop II dispenser (GC biotech, Netherlands). The real-time PCR, 12K quantstudio (Thermo Fisher Scientific Inc., CA, USA) was used to run the qPCR reaction. The Expression suite software v1.0.3, SDS 2.4 and RQ manager 1.2.1 software was used to analyse the raw data provided by the instrument.

The selection of CRSwNP risk genes for Paper IV was based on our findings in Paper III. All of the selected genes were top hits in our INRICH and eQTL analyses of NP target genes implicated in this study. Blood eQTL data from Westra et al. [103] together with our findings from Paper III were used to study SNP variations associated with gene expression patterns. All eQTL SNPs for each gene of interest were extracted and classified according to the direction of their regulatory effect (up-regulating or down-regulating). The frequency of the allele bearing the reported regulatory effect was then determined in our GWAS cases and controls.

The Delta-Delta CT ($\Delta\Delta$CT) relative quantification method was used for analyses of gene expression data [106]. This method normalizes expression of target genes to reference genes, and then compares this value between case and control samples (the $\Delta\Delta$CT value). The threshold cycle (CT) or the cycle of quantification, is the PCR cycle when the amplification reaches a set threshold. Calculation of delta CT is done by finding the difference between the cycle of quantification for the target gene compared with that of the reference gene. Significance between the mean delta CT values of the controls compared to the mean delta CT of cases was calculated using a t-test. Linear regression was used to analyse gene expression of specific alleles in the NP and control groups separately and together with allele count as predictor and compared to the eQTL blood dataset. All statistical analyses were conducted in R or SPSS with a p-value <0.05 considered to be significant.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Results

A total of 410 first-degree relatives of 368 patients with nasal polyps (one relative each for 162 patients and two relatives of different sex for 124 patients) were recruited; we were unable to recruit a relative for 82 patients. 55 of the relatives had nasal polyps themselves (38 men and 17 women). No family had more than two individuals with nasal polyps.

The prevalence of nasal polyps among the families with participating relatives was 19.2% (55/286) with a 95% CI of 14.7–23.8. When the prevalence is calculated among all relatives instead of families the prevalence was 13.4% (55/410) with a 95% CI of 10.1–16.7. Male relatives had a higher prevalence than female (20% and 7.7%, respectively; $p<0.001$). There was also an increased prevalence with higher age ($p<0.001$).

A previous study on 1387 individuals drawn at random from the general population, stratified for age and sex, of the same geographic area found a prevalence of nasal polyps of 2.7% [7]. When the two studies are compared, the first-degree relatives of nasal polyp patients had an almost five times higher relative risk (RR) of having nasal polyps themselves (RR=4.9; 95% CI 3.3–7.3). Both male relatives (RR=5.3; 95% CI 3.3–8.5) and female relatives (RR=4.5; 95% CI 2.2–9.3) had a higher gender-specific RR.

Discussion

Previous studies on the heredity of nasal polyps have utilised questionnaires to obtain a family history from patients with the disease and the investigators did not perform an endoscopic investigation of the nasal cavity to confirm or rule out the condition, which is necessary for diagnosis. This method has a clear risk of both under- as well as overestimation of the importance of any possible genetic factors involved in the pathogenesis. However, at least two of these studies have found an increased prevalence of positive family history when comparing cases and controls [17, 62]. Other studies have documented the prevalence of positive family history among patients with nasal polyps without comparing them to a control group [18, 63]. Paper I is the first study performed with a study group solely consisting of relatives of patients with CRSwNP and the first study on the heredity of this condition where the presence of CRSwNP

is determined using nasal endoscopy, enabling a more accurate assessment. The marked increase in risk among the first-degree relatives to patients with CRSwNP strengthens the results from previous research in the field and shows that heredity and genetics are of importance in the in the pathogenesis of this disease. Even though the lifetime risk of developing nasal polyps yourself when you have a first-degree relative with this condition cannot be calculated with this data, the increase in nasal polyp prevalence with a higher age of the relatives makes it likely that this risk is higher than 13%.

3.2 PAPER II

Results

Patients with CRSwNP were more often male and had a higher age (69% males, mean age 57 years) than controls (48% males, mean age 49 years) (Table 1). Both of these differences were statistically significant in both the univariate and the multiple logistic regression analyses (OR 2.86, 95% CI 1.98–4.14 and OR 1.03, 95% CI 1.02–1.04, respectively) (Table 2).

Subjects with CRSwNP had daily symptoms of nasal blockage (55.3% vs. 9.2%), sneezing (12.3% vs. 4.4% and nasal secretion (27.0% vs. 6.2%) significantly more often than controls. Impaired sense of olfaction (76.6% vs. 14.4%) and parosmia (8.2% vs 3.7%) was also more frequent among patients than controls (Table 1). All nasal symptoms had a significant OR in the univariate analysis but only nasal secretion, nasal blockage and impaired sense of smell remained significant in the multiple logistic regression analysis (Table 2).

The prevalence of asthma (54.5% vs. 9.9%), (sensitivity to cold 31.3% vs 14.4%) and daily cough (11.2% vs. 3.8%) was higher among patients with CRSwNP compared to controls (Table 1). Only asthma was significant in the multiple logistic regression model even though all three were significant in the univariate analysis (Table 2).

Smoking was more frequent among controls (14.2% current smokers) than patients (4.6% current smokers) (Table 1). Being an ex-smoker or a current smoker were significant factors when using never having smoked as a reference in the univariate analysis but the multiple logistic regression analysis showed that only current smoking was significant (Table 2).

Table 1. Respiratory symptoms in 367 patients with chronic rhinosinusitis with nasal polyps (CRSwNP) compared with 1349 polyp-free individuals from the general population.

	Patients with CRSwNP (N = 367)		**Controls (N = 1349)**		
	N	**%**	**N**	**%**	**p-value[1]**
Daily nasal secretion	99	27.0%	83	6.2%	<0.001
Daily nasal blockage	203	55.3%	124	9.2%	<0.001
Daily sneezing	45	12.3%	59	4.4%	<0.001
Impaired sense of olfaction	281	76.6%	194	14.4%	<0.001
Parosmia	30	8.2%	50	3.7%	<0.001
Daily cough	41	11.2%	51	3.8%	<0.001
Asthma	200	54.5%	134	9.9%	<0.001
Cold air sensitivity	115	31.3%	190	14.4%	<0.001
Smoking					
Never	189	51.5%	756	56.0%	
Ex-smoker	161	43.9%	401	29.7%	<0.001
Current smoker	17	4.6%	192	14.2%	

[1] Comparison of the prevalence/ proportion between the groups by Chi-2 test.

Table 2. Odds ratios (ORs) and 95% confidence intervals (CIs) based on univariate and multiple logistic regression, with polyps as outcome.

Factor	OR with 95% CI Univariate model	OR with 95% CI Multiple logistic regression model
Male sex	2.35** (1.84–3.00)	2.86** (1.98–4.14)
Age	1.03** (1.02–1.04)	1.03** (1.02–1.04)
Symptoms		
Daily nasal secretion	5.63** (4.10–7.76)	1.73* (1.05–2.85)
Daily nasal blockage	12.23** (9.27-16.12)	7.62** (5.13–11.32)
Daily sneezing	3.06** (2.03–4.59)	0.97 (0.51–1.84)
Impaired sense of olfaction	19.45** (14.62–25.88)	9.56** (6.76–13.53)
Parosmia	2.31** (1.45–3.69)	1.44 (0.69–3.02)
Daily cough	3.20** (2.08–4.91)	0.68 (0.33–1.38)
Asthma	10.86** (8.27-14.25)	9.91**(6.54-15.01)
Cold air sensitivity	2.78** (2.13–3.64)	0.77 (0.50–1.20)
Ex-smokers[1]	1.61** (1.26–2.05)	1.12 (0.78-1.62)
Smokers[1]	0.35** (0.21–0.60)	0.37* (0.19–0.72)

*statistically significant at the 5% level, **statistically significant at the 1% level
[1] OR calculated with never-smokers as reference group

Discussion

Several previous studies have reported that patients with CRSwNP are more likely to be males [7, 8, 11] and of higher age when compared to controls [6, 7, 8] and the data in Paper II is in agreement with these results. In other articles, symptoms reported by subjects with CRSwNP have included nasal blockage, altered sense of taste and/or smell, anosmia, rhinorrhoea and the need to blow one's nose [17, 23]. The subjects in Paper II reported symptoms similar to those in these previous studies. Subjects with asthma have been shown to have a higher prevalence of CRSwNP [5, 7, 9] when compared to subjects without asthma. Conversely a higher prevalence of asthma has been found among patients with CRSwNP when compared to subjects free of this affliction [17, 18], Paper II shows comparable results. Studies that investigate the role of smoking in CRSwNP have found varying results: one study found that smoking was less common among patients with CRSwNP than predicted when comparing the data to a national census [11] while another study found that smoking was more common among patients with CRSwNP than controls [107]. Paper II found that there were fewer current smokers among the subjects with CRSwNP than there were in the control group. It seems unlikely that smoking protects against CRSwNP, this association is more likely to represent another type of relationship, e.g. that smoking could be perceived as less enjoyable if you have CRSwNP.

When evaluating symptoms from patients with CRSwNP or any other disease from the sinonasal system it is important to remember that symptoms from the upper airways are common among the general population. Therefore, using a control group from the general population for comparison, as in Paper II, would make the results more robust and allow for testing in statistical models such as a multiple logistic regression model to strengthen them even further. Even though this study found similar results to studies that came before it, this is the first study that has compared the symptoms of subjects with CRSwNP to those of a control group or tested them in a multiple logistic regression model, therefore strengthening the results.

3.3 PAPER III

Results

After quality control the data set consisted of 782 individuals, 406 with nasal polyps and 376 healthy controls as well as 233 409 SNPs. rs4629180 was the most significant SNP in the DFAM analysis with a p-value of 1.47×10^{-6}, rs2491026 was the top-ranking SNP in the EMMAX analysis with a p-value of 0.00014.

The INRICH analysis produced a list of 138 target CRSwNP genes after the top 20 gene-sets had been extracted from each of the four separate analyses.

36 of these target CRSwNP genes showed a significantly different mRNA expression level when comparing nasal polyp tissue and normal tissue from Plager et al. [90].

HLCS (empirical p-value 0.014), *HLA-DRA* (empirical p-value 0.02) and *BICD2* (empirical p 0.046) had significantly skewed distribution of eQTL after analysis of the Blood eQTL dataset. *VSIR* (empirical p-value 0.006), *HLCS* (empirical p-value 0.014) and *BICD2* (empirical p-value 0.016) were significantly skewed after analysis of the MuTHER eQTL dataset. *SLC5A1* was borderline significant in the same test with an empirical p-value of 0.052.

Discussion

Paper III was the first GWAS performed only on subjects with CRSwNP, the first GWAS on any variant of CRS that utilised individual genotyping and the first study on the genetics of CRSwNP to feature a combination of association testing and genetic linkage.

A suggested and commonly used level of genome-wide significance is a p-value lower than $5x10^{-8}$, even though none of the SNPs in Paper III reached that level of significance, post-GWAS analysis nonetheless revealed genes of potential interest and importance in the pathogenesis of CRSwNP. Of these genes, *HLA-DRA* has been implicated in CRSwNP in at least one previous study [108], *HLCS*, *VSIR*, *BICD2* and *SLC5A1* are potential new genes of interest in CRSwNP.

Even though *HLCS* and *SLC5A1* themselves have not been associated to CRSwNP before, their respective gene-products, Holocarboxylase synthetase and solute carrier family 5 (sodium/glucose cotransporter) member 1 (SGLT1) have indirectly been implicated via their effects on other proteins.

Holocarboxylase synthetase has in one study been shown to induce the expression of *RANTES* via biotinylation of heat shock protein 72 [109]. RANTES protein levels have in turn been correlated to the severity of disease among patients with CRSwNP [110] and using immunological staining, RANTES has also been detected to a higher degree in nasal polyp tissue when compared to tissue from the nasal mucosa of unaffected individuals [111]. In the database from Plager et al, *HLCS* is under-expressed in polyp tissue [90], this may seem counter-intuitive given that Holocarboxylase synthetase induces

the expression of a gene whose protein is positively correlated to CRSwNP severity and more often detected in nasal polyps compared to unaffected nasal mucosa. However, it is possible that the down-regulation of *HLCS* is a reaction rather than a cause itself or that this could represent some other type of interaction such as different expression levels in peripheral blood and nasal polyps among individuals with CRSwNP (this is covered briefly in the discussion of the results from Paper IV). The exact relationship between *HLCS*, *RANTES* and CRSwNP is unclear and could warrant further study.

Even though they were unable to confirm a direct relationship between SGLT1 and CFTR, one group of researchers could demonstrate data that suggests a positive substrate-cross regulation between the two proteins [112]. As mentioned in the introduction, mutations in *CFTR* can lead to CRSwNP as a monosymptomatic form of CF [57] and patients with CF often suffer from CRS [56].

When conducting studies on genetic association (such as a GWAS), accurate phenotyping is essential as wrongly classifying a phenotype-positive test subject as phenotype-negative could dilute the differences between cases and controls and potentially make the investigators miss markers of importance to the phenotype in question. Paper III uses a control group which solely consists of first-degree relatives to patients with CRSwNP, this is a necessity in our family-based design and beneficial in that this could make the study better equipped at finding potential rare variants than a GWAS that uses unrelated subjects [113]. Related individuals are also expected to be more similar in many chromosomal regions, when there are dissimilarities between related individuals these are more likely to be caused by CRSwNP than by general differences in the population (i.e. population stratification). However, as shown in Paper I, relatives to patients with nasal polyps are more likely to have nasal polyps themselves and this risk increases with higher age. There is therefore a possibility that some of the controls in Paper III could develop nasal polyps later in life and therefore be misclassified as phenotype-negative (i.e. polyp-free) in Paper III, especially if they were relatively young at the time of investigation. Nonetheless, the mean age of the relatives in Paper III is 49.4 years and the prevalence of polyps among them is 13% which makes it unlikely that more than a few percent of them are falsely classified as polyp-free.

Another aspect of phenotyping when it comes to CRSwNP is that the division of CRS into two subgroups based on whether or not the individuals have nasal polyps is likely to be an overt simplification of the actual pathophysiological situation. In all likelihood, there are subgroups of CRSwNP with different causes and interactions between genes and possible environmental factors.

Therefore, the subjects with nasal polyps in Paper III could possibly be a more heterogeneous group than anticipated with regards to possible associations between SNPs and CRSwNP. This could in turn have decreased the power of the study. Nonetheless, little is known regarding the classification of nasal polyps and CRS into subgroups other than CRSwNP and CRSsNP at this time. Paper III therefore focused on the phenotype CRSwNP itself without attempts at further subdivision.

3.4 PAPER IV

Ten target genes were used for the analysis of gene expression and *YWHAZ* was used as reference gene. *BICD2*, *CPEB3*, *HLCS* and *NDUFS5* were nominally significant, *CPEB3* and *NDUFS5* were significant after correction for age and sex. After Bonferroni correction only *NDUFS5* was significant. All significant genes showed an upregulation in nasal polyp patients vs controls (Table 3).

Table 3. Gene expression in blood from Nasal Polyp patients versus controls with YWHAZ as reference gene. The Delta-Delta CT (ΔΔCT) relative quantification method was used determine the mRNA levels of target genes relative to a reference gene. The p-value was calculated using the independent sample t-test for equality of means.

Gene	P-value	P-value Age and sex	Mean Ct Diff	Std. Error	Lower	Upper	p-value[c]	FC	% change	NP vs Control
NDUFS5	0.002	0.005	0.60	0.19	0.23	0.97	0.022	1.52	52	UP
CPEB3	0.008	0.047	0.68	0.25	0.19	1.16	0.088	1.60	60	UP
HLCS	0.01	0.18	0.50	0.21	0.89	0.92	0.11	1.42	42	UP
BICD2	0.02	0.31	0.46	0.19	0.90	0.84	0.22	1.38	38	UP
PDGFD	0.06	0.28	0.44	0.24	-0.03	0.90	0.66	1.35	35	UP
VSIR	0.34	0.415	0.22	0.23	-0.67	0.23	1	0.86	-16	DOWN
HLA-DRA	0.39	0.68	0.11	0.15	-0.67	0.57	1	0.71	-8	DOWN
TIAM1	0.57	0.97	0.13	0.24	-0.35	0.61	1	1.18	9	UP
LYZ	0.67	0.90	0.11	0.29	-0.47	0.70	1	1.08	8	UP
HLADQB1	0.85	0.82	0.13	0.72	-1.34	1.60	1	1.09	9	UP

FC= Fold Change, NP= Nasal Polyps, P-value[c] = Bonferroni correction

Cases and controls were analysed together as well as separately and compared to the eQTL blood dataset [103]. *HLCS* and *PDGFD* were significant when nasal polyp patients were analysed separately; *TIAM1* was significant when the control group was analysed separately. Four genes (*HLCS*, *LYZ*, *PDGFD* and *TIAM1*) were significant when NP patients and healthy controls were analysed together. When mean ΔΔCt was compared between the alleles at specific SNPs, *HLCS* had four significant SNPs, *LYZ* and *PDGFD* had two each and *TIAM1* had one significant SNP. The allele which was highly expressed from our study was also highly expressed from the eQTL blood dataset and showed an upregulation after comparing the trend in mean ΔΔCt to the Z score from the blood eQTL dataset.

Discussion

All of the genes investigated in Paper IV were selected due to them being potential genes of interest implicated in Paper III. Instead of relying on expression data from other authors and test subjects (as in Paper III), expression analysis was performed on a subset of patients and their relatives from the current project. In Paper IV; *HLCS, CPEB3* and *NDUFS5* were found to be over-expressed in peripheral blood from patients with CRSwNP whereas they were under-expressed in the database from nasal polyp tissue used in Paper III [90]. One possible explanation to this discrepancy is that mechanisms started in peripheral blood or other sites in the human body could affect the paranasal sinuses and nasal cavity and conversely, processes that occur due to CRSwNP could provoke a response from other sites than just the affected tissue. Even though tissue from the nasal cavity could possibly have given different and perhaps more relevant results, using peripheral blood also allowed for the comparison of our results to data from an eQTL database (there is no eQTL database for nasal mucosa or nasal polyps). Of the genes implicated in Paper IV, only *LYZ* had been connected to CRSwNP prior to this thesis [114].

A unique aspect of this study on gene expression is that we are using the relatives of our cases as controls, thus possibly reducing the effects of population stratification.

4 CONCLUSIONS

After analysing the findings in this project, the following main conclusions have been drawn:

1. First-degree relatives of patients with CRSwNP have an almost fivefold increased relative risk of having nasal polyps themselves when compared to a control group drawn at random from the general population.

2. Daily symptoms of nasal secretion, nasal blockage and decreased sense of smell are more common among subjects with CRSwNP than among controls.

3. *HLCS*, *BICD2*, *VSIR*, *NDUFS5*, *CPEB3*, *PDGFD*, *TIAM1* and *SLC5A1* are potential new genes of interest in CRSwNP. *HLA-DRA* and *LYZ,* which have been previously implicated in the disease, are strengthened as interesting targets for further research.

4. *NDUFS5*, *CPEB3*, *HLCS* and *BICD2* are upregulated in peripheral blood samples from patients with CRSwNP when compared to controls.

5. *HLCS*, *LYZ*, *PDGFD* and *TIAM1* showed significant differences between carriers of different genotypes when comparing allelic expression among patients with nasal polys and/or controls.

5 FUTURE PERSPECTIVES

There are several ideas that follow after establishing that a condition has a hereditary component. One of which is that it makes host factors a more valid target for future research and sparks an increased interest in genetic factors as important drivers in the pathogenesis. An end-goal could also be developing a more tailored medical and/or surgical treatment for a group of patients where no treatment can offer a satisfying long-term result as of today. However, a more immediate benefit could be spreading the information regarding the heredity of CRSwNP among patients, their relatives and physicians.

Due to the increased risk of CRSwNP among relatives, patients with CRSwNP should be informed to advise any relatives who develop long-term nasal secretions, nasal blockage or a decreased sense of smell to contact their family doctor and schedule an appointment, preferably including nasal endoscopy.

Similar to this, physicians should be educated to be more suspicious of nasal polyps when relatives of patients with CRSwNP develop these symptoms and either themselves examine the patient's nasal cavity with an endoscope or refer the patient to an otorhinolaryngologist who will. This is especially true in male patients and/or patients of higher age.

With regards to future genetic research targets, this thesis adds information to a foundation laid by other researchers. *HLA-DRA* and *LYZ* have been suggested as genes of interest prior to these papers and are reinforced as such after this project. *HLCS*, *BICD2*, *VSIR*, *SLC5A1*, *NDUFS5*, *CPEB3*, *BICD2*, *PDGFD* and *TIAM1* are genes that have not been implicated in CRSwNP before and could warrant further investigation.

These potential future studies could include e.g. candidate gene studies in other populations, studies of gene expression from either nasal polyp tissue or blood or studies that explore eQTL further. At the time of writing, there is no eQTL database based on tissue from the nasal cavity or nasal polyps and the creation of such a database could possibly help future projects exploring gene expression or eQTL.

Genetic linkage has not been explored in patients with CRSwNP prior to thesis and investigating this field further, either on its own or in combination with genetic association may be beneficial, not least with regards to discovering potential rare variants associated to CRSwNP.

Since the three different GWAS performed on either CRS or CRSwNP utilise study populations of a mainly European descent, similar studies performed on populations with a different genetic background could help highlight potential similarities or differences between the study groups.

Another interesting possible use for genetic information is their potential as biomarkers for subtypes of CRSwNP. Even though little is known about what having an over- or under-expression of a certain gene or a risk-allele of a particular SNP means on a pathophysiological level there is a possibility that these markers could be used to differentiate patients into to subgroups with different responses to specific therapies. The main treatment options offered to patients with CRSwNP today; topical steroids, systemic steroids and surgery, either target inflammation in general or the diseased tissue itself in a rather unspecific manner and a meaningful and more detailed subtyping of CRSwNP could hopefully enable a more tailored and subtype-specific treatment with potentially better outcome for these patients.

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