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University of Dublin, Trinity College

MD Thesis

Oesophageal inflammation and tumourigenesis – Inherited Variation in an Irish population

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M.B.B.S. (Hons) MRCSI

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DECLARATION

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Mahwash Babar

Mahwash Babar

Summary

Cancer is a major medical challenge of this era and early diagnosis of cancer is critical to curative therapy. The search for biomarkers that may indicate the risk or presence of cancer is ongoing in thousands of laboratories around the world. Individual variability of DNA sequence distinguishes one person from another and have been used to search for biomarkers for cancer susceptibility.

Oesophageal cancer is the eighth most common cancer in the world and in Ireland its incidence is higher than in the rest of the European Union. Of the two main histological types of oesophageal cancer, Oesophageal Adenocarcinoma (OAC) incidence has increased by an alarming 500% in the last 3 decades in Ireland. The projected increase by 2020 in Ireland is estimated to be 84% for males and 39% for females. As the symptoms due to oesophageal cancer appear at a late stage, the overall 5 year survival is low and post operative morbidity is high. This is an issue worthy of concern and calls for increased research effort directed at finding ways of early diagnosis.

OAC develops on a background of reflux oesophagitis and Barrett's Oesophagus (BO) and there is evidence to suggest the role of acid and bile induced inflammation in its pathogenesis. OAC is also increasingly being recognized as a multi factorial disease whereby both environmental and genetic factors may be crucial. Therefore, the aim of this study was to further the understanding regarding the genetic background of oesophageal disease. We investigated the inheritance of polymorphisms in genes regulating the function of cytokines involved in inflammation, anti-tumour immunity (Interleukin -18 and Interleukin -18 Receptor Accessory Protein, IL-18 and IL18-RAP) and also genes involved in bile acid and

xenobiotic metabolism (nuclear receptors PXR, RXR, FXR and Tribbles protein TRB3).

This was a case control study examining SNPs selected in the 6 genes on the basis of the ability of the polymorphisms to either alter gene expression, to change amino acid sequence or ability to tag a particular haplotype. Subjects were recruited as part of an international collaboration with four different groups defined as controls, refluxers, Barrett's oesophagus and oesophageal adenocarcinoma. A total of 1919 subjects were included in the study (Controls=1238, Reflux oesophagitis=230, Barrett's oesopahgus=224 and OAC=227). Allele, genotype and carrier status frequencies for each individual SNP were compared statistically between patient and control groups using the chi-square or fishers exact test. Haplotype frequencies were estimated from unphased genotype data using computational methods and were statistically compared between groups. Gene-gene interaction analysis was also carried out using crosstabulation.

Results from this study have revealed a number of statistically significant associations between genetic variation and oesophageal disease in the Irish population. *IL18* and *IL18RAP* show a significant association with the pre-malignant group Barrett's oesophagus and OAC for functional polymorphisms *IL18*-607rs1946518(Genotype frequency; OR 1.48, CI 1.08-2.04, p=0.01) and *IL18RAP*rs917997 (Genotype frequency; OR 0.58, CI 0.42-0.79, $p=5x10^{-4}$). The association was similar in the Barrett's and adenocarcinoma group as compared with the controls and reflux oesophagitis group and specific correlations of these phenotypes were also strongly significant when compared against each other as two groups (Genotype frequency; OR 0.54, CI 0.42-0.68, $p=1x10^{-7}$ for *ILA8RAP*). Haplotype reconstruction of the htSNPs in the *IL18* gene shows significant differences

in the overall pattern of haplotypes between the cases and the controls. A significant association between *TRB3* Q84R polymorphism and OAC was also demonstrated. This SNP was associated with susceptibility to adenocarcinoma but not Barrett's oesophagus (Genotype frequency; OR 1.49, CI 1.04-2.14, p=0.02). No significant differences in allele and genotype frequencies were observed for functional polymorphisms in the nuclear/bile acid receptors *PXR*, *FXR* and *RXR*. *FXR* rs476498 genotype was associated with OAC (OR 1.53, CI 1.06-2.20, p=0.01) and significant interactions were observed between specific *FXR* and *RXR* polymorphisms ($p<0.0001$).

These results show that inherited variation in genes that regulate inflammation and anti-tumour response system, some of which have been associated with altered activity, are associated with susceptibility to BO and OAC. In particular a strong association was seen between *IL18RAP* and oesophageal disease, at levels of statistical significance comparable to those demanded of genome wide association studies (GWAS) ($p=5.10^{-7}$), without significant multiple testing, which thus makes this a highly significant finding. The *IL18* gene also showed evidence of association, although this was much weaker. Recently, altered levels of serum IL-18 have been shown in OAC patients. In this study, we have identified a number of genetic variants which are significantly associated with oesophageal disease, including newly investigated variants which have not been studied previously in association studies of oesophageal cancer. Such identified genetic factors may in the future be utilised to help elucidate the complete pathogenesis of oesophageal disease and hence, assist in the developments of new effective treatments.

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LIST OF ABBREVIATIONS

ABCB1	ATP-binding cassette (ABC) transporters 1
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
BBC	Biobank controls
BMI	Body mass index
BO	Barrett's oesophagus
CA	Chenodeoxycholic acid
CAR	Constitutive androsterone receptor
CARD15	Caspase recruitment domain member 15
CDKN2	Cyclin-dependent kinase inhibitor 2
CHOP	CCAAT/enhancer binding protein (C/EBP)
CI	Confidence Interval
CNV	Copy number variation
CYP3A4	Cytochrome P450 3A4
DCA	Deoxycholic acid
DIP	Deletion insertion polymorphism
DNA	Deoxyribose nucleic acid
EDTA	ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme linked immunosorbent assay
FINBAR	Factors influencing Barrett's Adenocarcinoma relationship
FXR	Farnesoid X-Receptor
GIT/GI	Gastrointestinal tract/Gastrointestinal

GORD	Gastroesophageal reflux disease
GWAS	Genome wide association studies
H2RA	Histamine 2 Receptor antagonist
H4TF-1	Histone 4 transcription factor-1
HGP	Human Genome Project
HIV	Human immunodeficiency virus
HNF4α	Hepatic Necrosis factor 4 α
HP	Helicobectar Pylori
htSNP	Haplotype tagging single nucleotide polymorphisms
HWE	Hardy-Weinberg Equilibrium
IFN_γ	Interferon_gamma
IL-1	Interleukin- 1
IL-18	Interleukin– 18
IL-18R1	Interleukin -18 Receptor 1
IL18RAP	Interleukin 18 Receptor accessory protein
IL-1R1	Interleukin- 1Receptor 1
IRS-1	Insulin Receptor substrate -1
LA	Lithodeoxycholic acid
LD	Linkage Disequilibrium
LOH	Loss of heterozygosity
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MDR1	Multi drug resistance protein1
MHC	Major histocompatibility complex
MKK4	Mitogen-activated protein kinase kinase 7
MKK7	Mitogen-activated protein kinase kinase 4

NFκB	Nuclear factor kappa B
NF-AT	Nuclear factor of activated T-cells
NK	Natural Killer
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain containing 2
OAC	Oesophageal adenocarcinoma
OR	Odds Ratio
OSCC	Oesophageal squamous cell carcinoma
PI3K	Phosphatidyl Inositol 3 kinase
PIK	Phosphatidyl Inositol
PPAR-γ	Peroxisome proliferator-activated receptors-γ
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PXR	Pregnane X-Receptor
RO	Reflux oesophagitis
RXR	Retinoid X-Receptor
SLC9A4	Solute carrier family 9 A 4
SNP	Single nucleotide polymorphism
STAT-1	Signal Transducers and Activators of Transcription
TAMRA	6-carboxy-N,N,N,N'-tetramethyl rhodamine
T1D	Type 1 Diabetes
TET	6 carboxy-4,7,2',7'-tetrachloro fluorescein
TGF	Tumour Growth Factor
TNF	Tumour Necrosis Factor
TRB-3	Tribbles-3
TSG	Tumour suppressor genes
VNTR	Variable non-tandom repeat

Presentations

Jan 2009: Oral presentation at annual conference of **Society of Academic and Research Surgery**, Bristol, UK.

Nov 2008: Poster presentation at **American Society of Human Genetics, Philadelphia, Pennsylvania,** USA.

May 2008: Oral presentation at annual conference of **Association of Surgeons of Great Britain and Ireland** (ASGBI), Bournemouth, UK.

May 2008: Poster Presentation at **Digestive Disease Week**, San Diego, California, USA.

Sep 2008: Two oral presentations at annual meeting of **33rd Sir Peter Freyer Surgical Symposium,** Galway, Ireland.

Publications:

- *Ready for submission to Gastroenterology*

Genes of the Interleukin -18 pathway are associated with susceptibility to Barrett's Esophagus: Results from the FINBAR case control study.

Babar M.*, Ryan A.W., Turner G., Murphy S.J., Hughes A.E., Patterson C.C., Anderson L.A., Watson R.G., Johnston B.T., Comber H., McGuigan J., Murray L.J., McManus R., Reynolds J.V.

Department of Clinical Surgery, Department of Clinical Medicine and Dublin Molecular Medicine Centre, Dublin, Ireland.

- *Publications from related academic work (American Journal of Surgery)PMID 19306979*

Differential molecular changes in patients with asymptomatic long-segment Barrett's esophagus treated by anti-reflux surgery or medical therapy

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- *Diseases of the Esophagus PMID 19930402*

Pilot translational study of dietary vitamin C in Barrett's esophagus

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Modified Nyhus-Condon Femoral Hernia repair

Babar M.*, E. Myers, M. J. Hurley.

This thesis is dedicated to my husband, Afzal

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Chapter 1
General Introduction

1.1 Cancer

There has been tremendous progress in the understanding of both the pathobiology and molecular biology of cancer and studies focussed on cancer development may result in the identification of novel effective therapeutic targets. The word cancer is often used broadly to refer to many diseases that have a common feature, namely uncontrolled cell growth. It has been shown that cumulative genetic mutation load leads to cancer development (e.g. colorectal carcinoma) hence functional genomics offer a powerful approach to understand the mechanisms involved in oncogenesis [1]. The Human Genome Project (HGP) has achieved a major landmark in generating an inventory of all human genes, and has provided researchers with invaluable technological and data analysis tools [2, 3]. The next phase is that of functional genomics, which attempts to correlate gene expression pattern and the protein function in the cells or tissues.

To ensure the optimal management of these diverse neoplasms, a close collaboration among surgeons, radiation and medical oncologists, pathologists, radiologists and gastroenterologists in the form of a multi-disciplinary approach is essential. Screening as a mode of early detection of cancer and identification of high-risk individuals is the cornerstone of successful outcome. Carefully designed epidemiological studies have contributed immensely to our understanding of risk factors for cancer and final outcomes. However, because epidemiological studies are generally observational by design, unknown and unmeasured confounding variables can affect the interpretation of results and, therefore, huge resources and large sample sizes are needed for powerful studies.

1.1.1 Genetics of cancer

Studies over the past two decades have implicated genetics in the aetiology of cancer [4]. It has been defined as a multistage evolutionary process resulting from accumulation of genetic mutations in a person's lifespan, usually in somatic cells. Aggressive uncontrolled tumour growth occurs when the mutant cell acquires aggressive growth properties resulting in clonal expansion[5].

Mutations in three classes of genes are particularly important in tumourigenesis. These are oncogenes, tumour suppressor genes (TSGs) and DNA repair genes [6]. Oncogenes are defined as pathologically altered forms of normal cellular genes, called proto-oncogenes that regulate cell proliferation, cellular senescence, differentiation, programmed cell death and genetic repair [7]. The most common genetic changes that occur prior to tumourigenesis are single nucleotide changes in coding or regulatory regions of genes, translocations, amplifications, deletions, or heritable epigenetic processes and these processes may activate proto-oncogenes [8]. Gene amplifications, chromosomal rearrangements and mutations through structural alterations become more common as the tumour progresses leading to further impairment of normal cell cycle regulation and cell proliferation.

TSGs protect cells against malignant transformation. TSGs also control cell division but they differ from oncogenes in that they generally inhibit cell division in the absence of necessary growth conditions [9]. TSGs lose their ability to control cell division when both copies of the gene are damaged by mutation or chromosomal abnormalities i.e. two mutagenic hits are necessary – as first shown in the case of retinoblastoma by Knudson et al. in 1971 [10]. Unlike oncogenes, it is usually a loss of function of these genes that facilitates the development of malignancy. Sometimes a

mutation in one of the alleles is inherited through the germ-line cells and only one mutation (in the second allele) is required to allow cell proliferation and tumour development e.g. retinoblastoma gene.

DNA repair gene mutations have been implicated in a variety of human cancers [11]. Like the TSGs, the DNA repair genes may be inactivated in human cancers and are often key activators of TSGs. Indeed some well described TSGs are integral components of DNA repair pathways – for example BRCA-1 and BRCA-2 [12]. Inactivation of DNA repair pathways in tumour cells results in an increased rate of mutations in other cellular genes including proto-oncogenes and tumour suppressor genes [11].

1.1.2 Genetic Variation and Cancer

1.1.2.1 DNA Sequence Variation:

The HapMap project has shown that approximately 99.9% of the human genomic sequence is identical between unrelated individuals [13]. The remaining 0.1% variation (i.e. 1 in every 300 nucleotides on average) represents polymorphisms that underlie the phenotypic differences among individuals and races. The best documented and most common types of genetic variation are single nucleotide polymorphisms (SNPs). More than ten million SNPs have been documented in the human genome and presumably many more, rare or private mutations exist. Other less frequent types of polymorphisms are variable number of tandem repeats (VNTRs), deletion / insertion polymorphisms (DIPs or indels), duplications, translocations and inversions. Increasing focus is being paid to genetic variants called 'copy number variation' (CNVs) which are defined as alterations to the DNA due to a variation in

the number of copies of a sequence within the DNA. These recurrent although relatively rare duplications and deletions are increasingly associated with common diseases with a genetic component [14, 15]. The majority of the genetic variation is located in non-coding regions of the genomes / genes which are likely not to alter gene expression. A small component of genetic variation in the non-coding regions does, however, alter gene regulation either at the level of expression or the location or timing of expression [16]. However, polymorphisms present in the exons of genes may or may not alter the amino acid sequence, and hence function of the protein (synonymous or non-synonymous SNPs) [17].

1.1.2.2. Gene polymorphisms And Cancer:

To date, the majority of the gene polymorphisms implicated in cancer epidemiology are located in genes encoding for metabolic enzymes involved in phases I and II of chemical metabolism or those involved in the DNA repair, vascular growth, cell adhesion and the inflammatory response [18]. Cancers arise from the accumulation of inherited polymorphisms (i.e. SNPs and mutations) and sporadic somatic mutations in cell cycle, DNA repair, and growth signalling genes [19]. For instance members of certain ethnic groups (e.g. Ashkenazi Jews) have a higher risk of carrying SNPs in cancer genes such as BRCA-1, BRCA-2, or APC (adenomatous polyposis coli) [20]. These SNPs confer an increased risk of developing breast, colon, prostate, or ovarian cancers [20]. Somatic polymorphisms such as those in the p53 gene, have been shown to influence both clinical outcome and response to treatment[21].

1.1.2.4. The HapMap Project:

Genetic association studies can use direct or indirect methods of testing. The former involves direct typing of a putative disease causing mutation, while indirect methods are based on the ability of a non-involved marker to detect association of a disease-causing variant. Co-inheritance of the marker variant with the disease susceptibility variant, more often than expected under the principle of independent assortment is termed as Linkage Disequilibrium (LD). Haplotype analysis of candidate susceptibility genes or regions is a standard approach in human genetics research and is based on the empirical finding that most regions of the genome are present as a small number of haplotypes in defined populations such as European Caucasians. This eliminates the need for testing all SNPs and is the basis of the use of haplotype tagging SNPs. This has been performed for several diseases e.g. cystic fibrosis and diastrophic dysplasia [22, 23]. Sequence analysis of the human genome has identified a vast number of SNPs, raising the expectation that this wealth of new information will allow, in the area of cancer, the identification of novel low-penetrance tumour susceptibility genes that can be used to estimate an individual's cancer risk [24]. The International HapMap Project was launched in October 2002 as a public, genome-wide database of common human sequence variation, with an aim to facilitate the genetic studies of clinical phenotypes [25]. The HapMap project has generated a detailed picture of linkage disequilibrium patterns, an invaluable tool to allow researchers to select a subset of all SNPs that capture most common genetic variation within a population, at however a fraction of the cost. Thus, instead of genotyping all ten million 'common' SNPs, the current technology platforms can genotype 500,000 to 1 million SNPs with little loss of information. This revolutionary approach has allowed much more efficient and comprehensive genome scans to identify regions with genes that affect cancers (and other common complex diseases).

However, it should be noted that rare genetic variants, which are increasingly associated with a number of inherited complex diseases (e.g. schizophrenia), are not well represented on these platforms[26] ..

1.2 Oesophageal adenocarcinoma and Barrett's oesophagus

An old saying in China is:

"A person who develops difficulty swallowing in autumn would not see winter"

Awareness about a disease of the food pipe leading to obstruction of the food passage and starvation dates back to Galen's time in the 2nd century. Oesophageal cancer has been described by the Canon of Avicenna and a thousand years ago, in the high incidence areas of Iran, cancer of the oesophagus was a known malady [27]. Attempts to treat oesophageal cancer were made as early as the beginning of the 19th century. Frank Torek performed the first oesophagectomy in 1913 and in the 1930s the first successful 1-stage transthoracic oesophagectomy was performed with reconstruction [27].

Oesophageal cancer remains one of the leading causes of cancer mortality in men, and is one of the top five causes of cancer-related mortality in black males [28]. The 5-year survival rates for oesophageal cancer have tremendously improved, almost doubling over the last four decades. However, compared with the survival rates from other cancers such as liver, lung and pancreas, oesophageal cancer survival is still one of the poorest. The 5-year survival rate for white patients has increased from 5% to 13% [28]. Fifty years ago, the commonest malignancy affecting the oesophagus was

squamous cell carcinoma. Over the last three decades, however, the incidence of adenocarcinoma of the oesophagus has increased in many countries at a rate that exceeds that of any other malignancy [29-33]. The precise reasons for the rapid increase in the incidence of this tumour remain unclear. The striking geographical distribution and the racial disparity in the incidence of oesophageal cancer suggest environmental roles in addition to genes as causative factors for the disease.

1.2.1 Epidemiology of Oesophageal Cancer and Barrett's adenocarinoma

Oesophageal cancer constitutes 7% of all gastrointestinal cancers [34], is the third most common gastrointestinal tract (GIT) cancer, the eighth most common cancer in the world (sixth most common in men and ninth most common in women) with about 300,000 cases diagnosed each year and the sixth most common cause of cancer-related death (5.5% of cancer-related deaths) [35]. Of particular concern, oesophageal cancer is fifth in ranking in developing countries[35]. Compared with all gastrointestinal (GI) cancers, the incidence of oesophageal cancer is highly variable depending upon geographical region (there is > 200-fold difference in the incidence between the high and low risk areas of the world). Whereas Oesophageal Squamous Cell Carcinoma (OSCC) is the commonest pathological subtype in Asia and Middle East, the incidence of OAC has increased over the last 3 decades in individuals of European descent. A rise of 9 – 16% per year has been reported in Scandinavian countries and an annual rise of 20% has been reported in Australia and the USA (in fact there has been 350% rise since 1970 in the two continents) [36]. Developed countries of Europe and North America have a higher incidence of oesophageal adenocarcinoma as compared with OSCC. In fact oesophageal adenocarcinoma has

the fastest growing incidence in Switzerland, Sweden, Norway, Denmark, England, Canada and the US [37].

Overall, in the populations of European descent, while the incidence of OSCC has remained stable over the last three decades, the incidence of OAC has risen from 0.7 to 3.2 per 100,000 during this period. [30]. In Europe the incidence of OAC now roughly equals that of OSCC.

OAC is more common in males with the incidence peaking at 75-80 years of age (rarely occurring before the age of fifty years). Compared with females, men have a tenfold greater risk of developing OAC [38]. The large increase in OAC has been associated with increasing recognition of BO and GORD [39].

1.2.1.1 Oesophageal Cancer in Ireland

In Ireland, rates of oesophageal cancer are higher than in the rest of the European Union in both males and females with a male / female ratio of incidence at 2:1[40] compared with 4:1 in the rest of Europe. In Ireland, the urban areas have significantly higher rates of incidence of oesophageal cancer as compared with rural areas. The annual average incidence/ death rate of oesophageal cancer in males is 185/191 while it is 120/118 in females [41]. The projected increase by 2020 is estimated to be 84% for males and 39% for females [42]. On average, females are estimated to have approximately a 1-in-240 chance of developing this cancer by age 74, and males have 1-in-103 chance. In Ireland, the occurrence of oesophageal cancer below age 35 is extremely rare, but the rate of diagnosis increases after age 45. Mean age of diagnosis is 74 years for females and 68 years for males [43].

Compared with other cancers, such as breast, lung or colorectal cancers, the incidence of oesophageal cancer is far less common. Nevertheless, because early

detection is difficult and usually patients present at an advanced stage with a poor prognosis, it remains a medical and scientific challenge. In the European Union, national programmes like effective screening and improved disease awareness have either reduced or prevented an increase in mortality (per 100,000) from most cancers (barring lung cancer in women) in the last 50 years. Unfortunately, this has not been the case in oesophageal cancer, partly because of widespread alcohol abuse and the comparatively lower incidence and subsequent difficulty in achieving a large enough sample size for powerful scientific studies.

1.2.2 Risk Factors

The epidemiology of oesophageal cancer has provided invaluable insights into genetic, environmental and other nutritional factors that may modify the susceptibility of the oesophageal epithelium for neoplastic development.

1.2.2.1 Age

There is an age related increase in the incidence of OAC [44]. The incidence is very low under the age of 40. After age 40, an increase in incidence is seen with each decade of life.

1.2.2.2 Gender

OAC is more common in males than females for all races and ages with a male: female ratio 6:1 [44]. These gender differences have been suggested to be attributable to alcohol intake and smoking.

1.2.2.3 Ethnicity

The incidence of OAC in the US White population compared to African-Americans is 3:1 [45]. However, mortality from oesophageal cancer is higher among blacks than among all other races [46]. African-Americans also tend to present with more advanced stage of disease and have poorer survival for similar stages of disease as compared with their white counterparts [45].

There is evidence of heritability in oesophageal cancer. Heredity is a well-defined risk factor for oesophageal cancer in the West and in countries like China and Japan with heritability index reported as high as 40% among first degree relatives [47, 48].

1.2.2.4 Obesity

Obesity has been shown to be an independent risk factor for oesophageal cancer[49, 50]. There are numerous mechanisms whereby obesity may confer an increased risk. First it predisposes to gastrooesophageal reflux by increasing the intra-abdominal pressure. Second it may explain the changes in the dietary habits of the individual, predisposing to reflux or increased cancer risk. An association between BMI and OAC has been shown by Lagergren et al. and many others[49, 51, 52].

1.2.2.5 GORD and Barrett's Oesophagus (BO)

BO is a metaplastic change from normal oesophageal squamous epithelium to intestinalized columnar epithelium. BO is an important risk factor for OAC. It reflects an injury to oesophageal mucosa as a result of acid and bile reflux (Figure 1.5) and is associated with GORD [53]. Chow et al. reported a two-fold increase in the risk of developing OAC among patients with a history of GORD more than one year before the diagnosis of cancer [39] while an OR of 7.7 (95% CI, 5.3-11.4) was observed in a

population based study when the frequency, severity, and duration of reflux were increased [54].

1.2.2.6. Medications

Certain medications like anticholinergics can relax the lower oesophageal sphincter, thereby potentially provoking reflux[55]. Calcium blockers can also relax the lower oesophageal sphincter and also can have an antiapoptotic effect [56]. Other drugs implicated include H_2 receptor antagonists (H2RA) but some studies have found no association with either[39]. A protective effect of aspirin has been observed, the most likely mechanism being its immunosuppressive/anti-inflammatory effect by inhibition of prostaglandin synthesis [57].

1.2.2.7 Helicobacter pylori (HP)

Most evidence with regards to HP in GORD and oesophageal cancer is based on epidemiological studies which suggest that eradication of HP may result in the onset of GORD [58]. This is supported by the fact that the downward trend noted in the Western world in ulcer disease has been accompanied with a rise in GORD and OAC [59]. Interestingly, most data to date correlating the two diseases is indirect and conflicting[60].

Figure 1: Potential factors contributing to development of oesophageal cancer

1.2.3 Molecular Biology of the metaplasia-dysplasia-adenocarcinoma sequence in the oesophagus

The rapid rise in incidence and striking geographical variation in oesophageal cancer reflect its multifactorial aetiology. Environmental factors suggested to be implicated in oesophageal cancer have already been discussed. The molecular events involved in development of oesophageal cancer are illustrated in Figure 1.12. The development of oesophageal cancer is a multi-step process involving genetic phenomena that result in key abnormalities of cell cycle regulation, intercellular adhesion mechanisms and growth factor activity [61].

1.2.3.1 p53 mutations

p53 TSG, the most commonly mutated gene in cancers, is mutated in about half of all oesophageal cancers [62]. Only 5% of patients with Barrett's metaplasia without dysplasia were shown to be positive for p53 mutation, whereas 15%, 45%, and 53% of subjects with low-grade dysplasia, high grade dysplasia, and OAC respectively were positive for p53 overexpression [63].

1.2.3.2 Epigenetic mechanisms

Epigenetic mechanisms such as hypermethylation can lead to suppression of gene expression. Altered methylation patterns have been reported in approximately 92% of OAC and 39.5% of BO. The p16/CDKN2, a growth suppressor gene on chromosome 9, that can regulate S-phase to G-phase transition in the cell cycle is the gene most commonly implicated in altered regulation due to epigenetic mechanisms[64]. In one report of hypermethylation assay of 20 genes, a greater proportion of hypermethylated genes were found in BO and OAC with advanced stage and poor differentiation [65].

1.2.3.3 Loss of Heterozygosity (LOH)

Loss of heterozygosity in a cell represents the loss of normal function of one allele of a gene in which the other allele was already inactivated. This occurs by deletion of all or part of the functional copy of the gene, hence the term – loss of heterozygosity (LOH). The molecular progression of benign Barrett's metaplasia to invasive cancer appears to occur due to accumulating genetic mutations within the oesophageal mucosal cells. A LOH has been shown in chromosomes 5q, 9p, 13q, 17p, and 18q with loss of 17p suggested to be an early event in Barrett's progression to OAC[66].

The germline mutations andalterations in the cell cycle pathways including cyclin D1 overexpression have been suggested to promote cellular proliferation and increased susceptibility to OAC.

1.2.3.4 EGFR

The EGFR family comprises erbB-1, erbB-2, erbB-3, and erbB-4, all of which are tyrosine kinase receptors. The binding of EGF or TGF-α to EGFR results in downstream signalling via MAPK and phosphatidylinositol 3-Kinase leading to increased DNA synthesis and cellular proliferation [67]. Over-expression of the ligands that bind EGFR, i.e. EGF and TGF-α have been shown in OAC [68]. Of these ligands, TGF-α is thought to be more important in dysplastic tissues where it promotes juxtracrine oligogenic stimulation through its incomplete cleavage of prepro TGF-α [69]. TGF-α overexpression is important in Barrett's progression to OAC [70, 71].

1.2.3.5 p73 Gene

A case control study carried out in this laboratory investigated the presence of noncoding *p73* compound polymorphism (denoted AT or GC- two closely linked

polymorphisms in ~ 100% linkage disequilibrium) in the 5′UTR of the *p73* gene. p73 is considered a member of the same family of proteins as p53. There were significant differences for AT/AT homozygotes between the OAC cases and controls (OR, 0.11; 95% CI, 0.02-0.6). The AT/AT homozygotes were much less prevalent in cases with a 9-fold reduced risk, suggesting that AT/AT homozygotes may be protected against the development of OAC [72].

1.2.3.6 Apoptosis

Oesophageal cancer evades the process of apoptosis to progress and metastasize. In Barrett's mucosa, a reduced apoptotic activity has been reported where a reduced number of apoptotic cells was observed in the upper zones in dysplastic and neoplastic glands as compared with benign metaplasia [73]. In the context of oesophageal cancer three molecules involved in apoptosis, namely Bax, Bcl2, and Fas, have previously been shown to be important[74]. p53 is a transcriptional activator of Bax gene whose product is a classic pro-apoptotic molecule. Bcl2 is an apoptosis suppressing gene of which Bax is a dominant inhibitor. In an OAC cell line, an inability of cells to translocate Fas, (an apoptosis mediating cell surface receptor) to the cell surface has been observed [75].

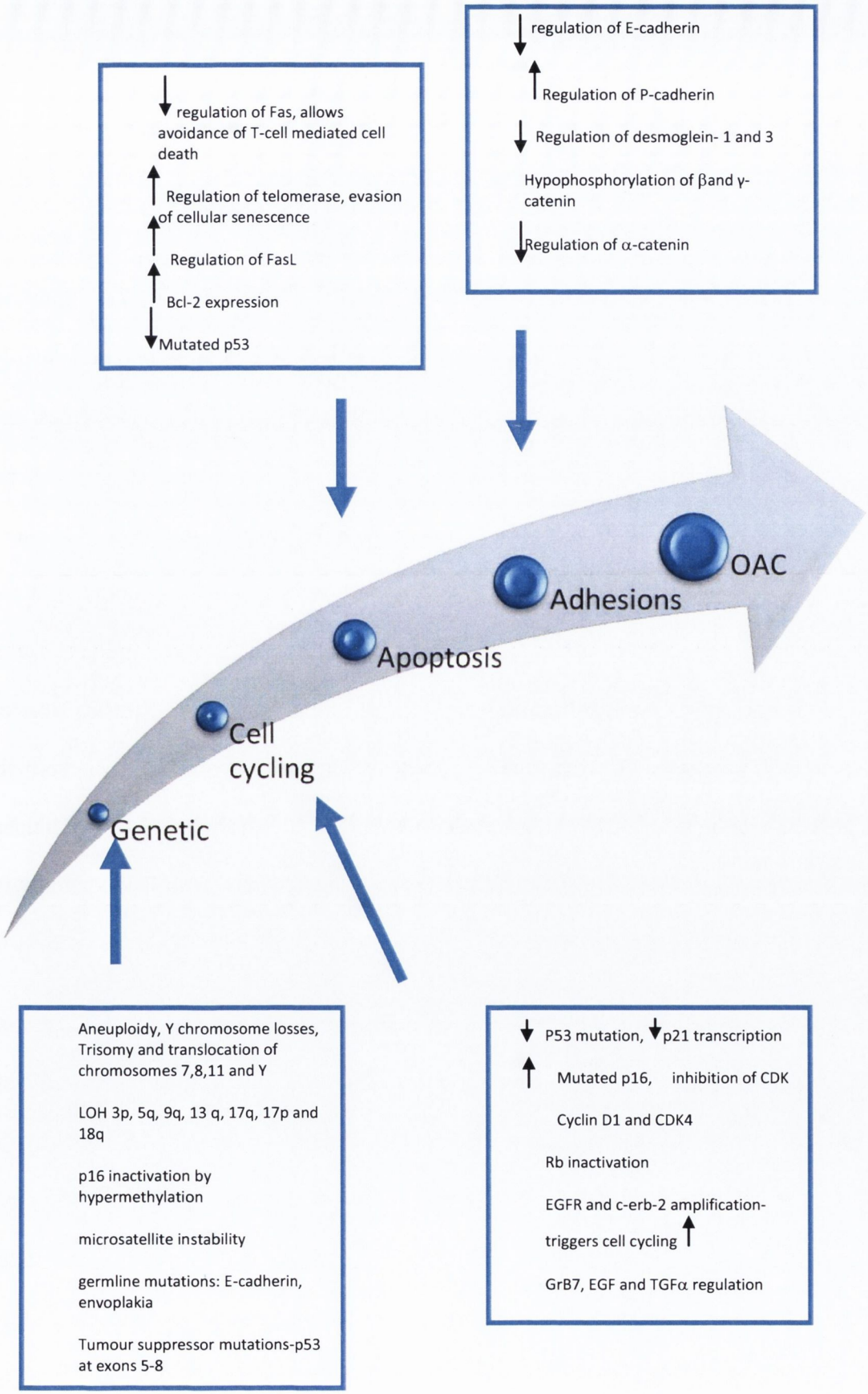


Figure 2: A summary of the potential molecular events involved in the pathogenesis of oesophageal cancer (Adapted from Gastrointestinal Oncology 2004, Editors Abbruzzese, J.L., Evans, D.B., Willet, C.G. and Preiser, S.F.

1.3.1 Immune system and cancer- Role of inflammatory cytokines

1.3.1.1 Immuno-editing and Immuno-protection in cancer :

Paul Ehrlich in 1909 was one of the first to propose that the immune system was a key player in identifying and eliminating nascent tumour cells, a hypothesis which was initially challenged but later widely accepted as cancer immune-surveillance hypothesis. The immune response comprises two main components, the cell-mediated and humoral response and there is compelling evidence to suggest that the T cell (cell-mediated response) plays a major role in the immune response against cancer[76, 77]. Robert D. Schreiber [78] used genetically engineered mice lacking a functional immune system and showed that lymphocytes and the immune stimulator, IFN-γ (involving STAT1 pathway), work together to inhibit the development of both spontaneous and carcinogen-induced tumours. In the human model, further support for the anti-tumour effect of naturally occurring immune responses comes from the increased incidence of cancer in immuno-suppressed transplant patients [78, 79]. Indeed, the incidence in transplant patients is significantly higher for cancers associated with or without viral infections, e.g. HIV [80, 81, 82].

However, this natural body defence is not perfect as some tumour cells escape identification and go on to cause cancer. These tumours have acquired less immunogenic properties by undergoing a process of immunoselection triggered by the actions of the immune system, a process referred to as 'immuno-sculpting or immune-editing' [78, 83, 84].This process is facilitated by proteins called tumour associated antigens, defined as normal or mutated proteins over-expressed by tumours [85]. The tumour associated antigens, like other antigens such as viral proteins, are presented by

MHC molecules and recognized by T cells. This process promotes or selects tumour variants with less immunogenic variants that are better suited to survive in an immunologically intact environment (tumours with reduced immunogenicity). Thus growing tumours (particularly those with genetic instabilities) can evade immunologic detection and elimination. [86](Figure 1.2)

1.3.1.2 Cytokines and oncogenesis

The history of cytokines dates back to almost half a century ago when host factors like inflammatory exudates were noted to induce fever[87, 88]. This was supported by studies on macrophages which demonstrated that antigen stimulated lymphocyte cultures produce macrophage activating factor [89]. Gradually a vast number of cytokines have been structurally identified and defined as protein molecules that possess lymphoproliferative and immuno-modulating activities. The considerable interest in the immunology and genetics of cytokines is due to the diversity of their functions. They act as families of protein messenger molecules produced by cells involved in differentiation, cell division, inflammation, immunity, fibrosis repair etc. Some cytokines have immuno-enhancing effects while others have immunosuppressive actions.

A precise regulation of all the genes of the genome is essential for the health of an individual. As cytokines are involved in nearly all biological processes, therefore their altered activity influences a multitude of pathological processes. Cytokine genes are highly inducible and very frequently polymorphic and since polymorphisms are frequently in regions of DNA that regulate transcription or posttranscriptional events, they may be functionally significant. Studies of such polymorphisms and cancer susceptibility and severity strongly implicate certain cytokine genes as cancer-

modifier genes, for example, in the context of non-Hodgkin lymphoma, systemic release of TNF and lymphotoxin contributes to the severity of the disease spectrum[90]. Interestingly, common inflammatory diseases like Rheumatoid Arthritis possess similar characteristic features of hyperproliferation, angiogenesis, and leukocyte infiltration as observed in developing tumours. Indeed, a variety of transcription factors that have been implicated in the inflammation-cancer sequence, such as NFkappaB, NF-AT, and AP-1 are involved in regulating cytokine gene expression [91, 92].

Of the pro-inflammatory cytokines, interleukin -1 (IL-1) family has been strongly implicated in carcinogenesis in both animal[93] and human models [94]. Local production of IL-1 has been shown to promote metastases in mouse models and secretion of these pro-inflammatory cytokines in the human multiple myeloma models promote tumour growth and resistance to therapy. [95] The proposed mechanism for these pro-carcinogenic cytokines is induction of a strong inflammatory response with accumulation of bacterial toxins and inflammatory mediators as shown by *IL1* gene cluster polymorphism studies in patients with pancreatic [94]and *Helicobectar pylori* induced gastric cancer. Thus, the members of the IL-1 family represent candidate cytokines and genes to investigate association with oesophageal disease pathogenesis.

1.3.1.2.1 Interleukin- 18 and cancer

Interleukin- 18 (IL-18) is a member of the IL-1 cytokine family. It is a multi-functional cytokine and is produced by a variety of cells, including T and B cells, monocytes, dendritic cells and macrophages. [96] IL-18 can induce pro-inflammatory cytokine expression, most notably IFN-γ. [97]

Besides its central function in innate and adaptive immunity, IL-18 has been identified both as a promoter and suppressor of oncogenesis. Through activation of an immune response, and particularly of Natural Killer (NK) cells, it promotes cell death and tumour regression [98]. In experimental cancer models, *IL-18* gene transfection into tumour cells has been shown to enhance both specific and non-specific anti-tumour responses [99, 100]. In the context of oesophageal cancer, Tsuboi et al [101] showed that IL-18 levels may be associated with biological features including stage of the disease. Thus the relationship of IL-18 with cancer is well known but appears complex and underlying mechanisms are currently the focus of investigation.

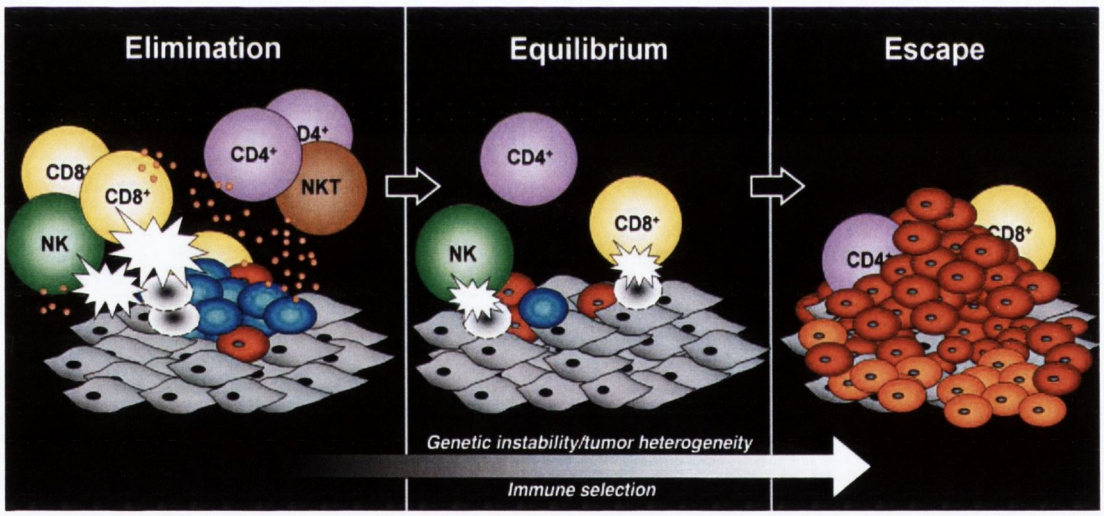


Figure 3: Cancer immune-editing

It encompasses three processes (a) Elimination represents cancer immunosurveillance (b) Equilibrium corresponds to the process of selective generation and promotion of the tumour variants by the immune system to enable survival against immune attack (c) Escape reflects immune-sculpting where tumour cells expand in an uncontrollable manner in the immunocompetent host. In (a) and (b), developing tumour cells are blue, tumour cell variants are red, underlying stroma are gray. In (c), additional tumour variants are orange. Different lymphocyte population as marked and the white flashes represent cytotoxic activity.

(Picture adapted from original publication in "Dunn, G.P. et al, Cancer immuno-editing: from immunosurveillance to tumour escape. Nat. Immuno. 2002 3 (11) pg 991-8").

1.3.2 Inflammation and the development of cancer

The association between inflammation and cancer was first described in 1863 by a biologist, Rudolf Virchow. He reported leukocytes in the neoplastic tissue clusters and suggested that the "lymphoreticular infiltrate" in fact represents the origin of cancer at the sites of chronic inflammation [102]. Tumours have also been described as non-healing wounds by Dvorak [103]. Inflammation involves a complex reaction to microbial, chemical, or physical agents in tissues, resulting in the influx of circulating leukocytes and connective tissue cells [104] which is a normal physiology process. However, when inflammation fails to resolve, chronic inflammation occurs which represents a risk factor for a range of diseases including cancer. The role of cytokines, chemokines and mechanisms of cell damage by pro-inflammatory molecules has already been discussed in the previous section. Hence the inflammation induced injury leading to carcinogenesis is well established in many solid organ cancers which are listed as follows in table 1.

Table 1: Inflammatory conditions associated with Cancer risk.

Inflammatory/infective stimuli	Malignancies
Papillomavirus	Cervical / Oropharyngeal Cancer
Human herpesvirus type 8	Kaposi's Sarcoma
Inflammatory bowel disease	Colorectal Cancer
Hepatitis virus (B and C)	Hepatocellular Carcinoma
Cigarette smoke, silica, asbestos	Bronchogenic Carcinoma
***Opisthorchis viverrini, Clonorchis sinensis* (Liver Flukes)**	Cholangiocarcinoma
Epstein-Barr virus	Non-Hodgkin Lymphoma Hodgkin Lymphoma Nasopharyngeal Carcinoma
Chronic cholecystitis	Gallbladder Carcinoma
Inflammatory atrophy of prostate	Prostate Carcinoma
Schistosoma hematobium	Bladder Cancer
Asbestos	Mesothelioma
HP induced gastritis	Gastric Cancer
HP	MALT Lymphoma
Pelvic inflammatory disease	Ovarian Cancer
Barrett's metaplasia	Oesophageal Adenocarcinoma

1.3.2.1 Inflammation-cancer sequence in Oesophageal adenocarcinoma: Pivotal role of NF-κB

There is compelling evidence that OAC occurs as a result of progressive inflammation induced injury to the oesophageal mucosa. Longstanding bile and acid reflux leads to replacement of lower oesophageal columnar epithelium by intestinal epithelium and this segment of specialised intestinal metaplasia is defined as Barrett's Oesophagus (BO) [105]. BO increases the risk of OAC by about 40- fold and is the only known malignant precursor of OAC [106]. A clear biological link between the cellular differentiation from hyperplasia, metaplasia, and dysplasia to adenocarcinoma is not

established yet, but it is generally believed that the refluxate induces the lower oesophageal mucosal cells to release inflammatory mediators such as cytokines and chemokines. This ultimately attracts the inflammatory cells including T-cell lymphocytes, neutrophils and activation of an important pro-inflammatory mediator nuclear factor kappa B (NF-κB). NF-κB upregulates the transcription and expression of a wide range of genes coding for cytokines, enzymes, apoptosis, proliferation and adhesion molecules which are involved in inflammation and tumourigenesis [107-109] and has been shown to be actively upregulated in OAC. Thus, a positive autoregulatory loop is established that can amplify the inflammatory response and increase the duration and intensity of chronic inflammation [110, 111].

1.3.2.2 Role of Bile acids in BO and OAC

Gastrooesophageal reflux disease is one of the major contributors to the development and increased incidence of OAC. A nationwide population-based case-control study performed in Sweden reported an odds ratio of 7.7 (95% confidence interval, 5.3-11.4) for adenocarcinoma among persons with recurrent symptoms of reflux, compared with controls; this increased to an odds ratio of 43.5 (95% confidence interval, 18.3-103.5) among patients with prolonged and severe symptoms of reflux [112]. The noxious stimuli responsible for GORD either arise from stomach (hydrochloric acid and pepsin) or the duodenum (bile acids and pancreatic secretions), however, mixed bile and acid reflux is more harmful to the oesophageal mucosa than acid reflux alone in humans [113, 114]. This is further supported by work done by Tselepis et al. who were able to induce proliferation in a model of Barrett's adenocarcinoma by intermittent unconjugated bile acid exposure (both Deoxycholic acid and Chenodeoxycholic acid) [115].

1.3.2.2.1 Role of Bile acid receptors in BO and OAC

Bile acid receptors are orphan nuclear receptors activated by bile acids, in particular chenodeoxycholic acid. These receptors have recently been identified and currently are a focus of interest in many gastrointestinal and immune related disorders. In fact, genetic polymorphisms in these receptors have been linked with inflammatory bowel disease, altered xenobiotic metabolism and liver disorders [116-118]. Increased expression of the xenobiotic receptors, Farnesoid X-Receptor, Retinoid X-Receptor (FXR and RXR) as well as CYP3A4 (a drug metabolizing enzyme upregulated by another nuclear receptor which also binds bile acids, Pregnane X-Receptor PXR/SXR) has been reported in oesophageal disease [119-122]. However, the contribution of bile acid receptors to the apoptotic processes and pathogenesis in BO and OAC is unknown.

1.3.2.3 Obesity, metabolic syndrome and Oesophageal disease

Obesity is an independent risk factor for OAC [51, 123, 124]. One of the mechanisms by which obesity increases the risk of adenocarcinoma could be increasing intra-abdominal pressure leading to greater gastrooesophageal reflux and associated transformation of distal oesophageal epithelium. Recent evidence has confirmed a strong association of OAC and BO with obesity, metabolic syndrome and disordered glucose metabolism [49]. Further research is currently ongoing to unravel the exact molecular link between obesity related inflammatory state and oesophageal carcinogenesis.

1.3.2.3.1 Role of TRB3 gene in metabolic syndrome

Tribbles is a family of proteins that play a key role in intracellular signalling pathways and a wide variety of physiological processes. In particular, they interact with other proteins including NF-κB and Akt, thus mediating the process of insulin sensitivity

and glucose metabolism [125-127, 128]. They have recently been linked to diseases like cancer and diabetes [126, 127] and currently are the focus of intensive investigation in an attempt to better understand their biology and function.

1.4 The design of case-control association studies

> ***"It will only be a matter of time before physicians can screen patients for susceptibility to a disease by analyzing their DNA for specific SNP profiles."***
>
> (NCBI_ http://www.ncbi.nlm.nih.gov/About/primer/snps.html)

Genetic association studies are one study design for the detection of genetic variation that is associated with common, inherited and usually multi-genic disease. Association studies generally are of two types; the family based study design or the case-control study design. For a case-control study, the principle is to detect a difference in allele frequency for a variant between cases and controls-this can be a direct association with a risk allele or an indirect association with a marker allele in LD with the true risk variant as explained above. The case-control study aims to estimate the risk that is associated with a particular genetic factor; in this case, exposures (i.e. presence of a particular genetic variant) are compared between groups of people with and without the disease of interest. The control group should be drawn from the same population whose cases are sampled. Appropriate sampling of the population should ensure that selected control individuals reflect the spectrum of allele frequencies and environmental exposures present in the overall population. It should be noted however, that the strict definition of random sampling of cases from a population can generally only be achieved for conditions where occurrence is

automatically registered (e.g. a cancer incidence register) [129] so that a random sample can be taken from anybody on that register; however, in practice, this is difficult to achieve.

For most case-control studies, the only feasible option is to recruit patients from individual hospitals or medical centres. In these situations, the control population should then be selected from the catchment area of the patient recruitment centre.

The odds ratio (OR) is the standard measure of the strength of a risk factor in a case-control study. This is defined as the odds of exposure among cases divided by the odds of exposure among controls. An OR of 1 means that individuals exposed to the risk factor (e.g. genetic variant) are at no increased risk of disease compared with those who are not exposed to the risk factor. An OR of >1 implies an increased risk and $0 < OR < 1$ means a protective effect. For multifactorial diseases risk factors conferring high ORs are generally rare, so an individual risk factor may not cause an absolute increase in prevalence of the disease.

Genetic association studies employ an indirect method of testing for association which relies on the ability of a marker to detect association of a disease-causing variant. This relies on the assumption that the marker variant will be more often co-inherited with the disease susceptibility variant than due to the phenomenon of linkage disequilibrium. Linkage disequilibrium (LD) therefore is defined as a lack of independence of assortment which is found to occur to varying extents between physically linked loci. LD occurs as a byproduct of the generation of new genetic variation, which arises as a result of mutational events on extant haplotypes. Therefore the new variant/mutation is fully linked to its neighbouring, preexisting alleles on the chromosomal environment or haplotype and will remain thus until

dislodged by genetic recombination. Therefore, closely linked alleles tend to stay in LD longer than alleles spaced farther apart and LD eventually decays as a result of homologous recombination. This simple representation is complicated by the observation that recombination does not occur at a linear rate along chromosomes but rather at discrete sites giving rise to the phenomenon of haplotype blocks, within which LD persists for longer than expected and which has important implications for association studies.

The most common statistical measures of LD between two markers are D and r^2. A number of measures of LD are available including the disequilibrium coefficient (D), D′ and r^2. D is equal to the difference between the observed and expected frequencies of the haplotype. However observable values of D are highly dependent on the frequencies of the constituent alleles in the haplotype, making comparisons between D values almost meaningless. Therefore D is usually expressed as the more standardized measure D′, which is calculated by dividing D by its maximum possible value given the allele frequencies at the two loci. D′=1 occurs if two SNPs have not been separated by recombination during the history of a sample (i.e are in complete LD). When D′<1, the ancestral haplotype has been disrupted. Another measure commonly used is the correlation coefficient between linked alleles, r^2. r^2 has the benefit of extracting useful information from allele frequencies at the constituent loci – thus for an r^2 of 1, both alleles in LD need to be fully linked and have the same frequency, which does not apply to D'.

Other important factors, along with LD, in the design of case-control studies are the frequencies of the disease-causing allele and the marker. Rare variants can reduce the power of statistical tests to detect association, and therefore common

variants are more susceptible to identification as disease-causing variants in the case-control association study design [129].

1.4.1 Haplotype based association

One major drawback with the analysis of single markers with disease association is that it does not detect specific combinations of neighbouring polymorphisms which reflect functioning units of the genome – haplotypes, and may therefore fail to detect genuine contributing factors to disease. As inferred, polymorphisms that are closely linked may frequently be inherited together as a unit, called a haplotype block. Haplotype information obtained from genotypes can better characterize the role of a candidate gene in the causation of a complex trait and, therefore, has become an important component of association studies.

Haplotype analysis is a comprehensive and informative analysis of the genetic variation in the region and it enhances the probability of detecting a disease association on the basis of interrogating the region rather than a single SNP or marker. This approach is also biologically relevant since it also takes into account the biologic function of genes, co-inheritance of loci and important interactions among proteins [130] and can increase the statistical power of the study by reducing the need to correct by multiple testing [131].

1.4.2 Haplotype tagging

The phenomenon of haplotype construction by LD has enabled researchers to conduct association studies by using only a few common haplotypes to characterize most genetic variation of a specific genomic region of the population [132, 133]. This observation of LD structure has allowed the identification of reduced sets of SNPs

that uniquely identify, or tag particular haplotypes [134]. These are referred to as haplotype tagging SNPs (htSNPs) and capture all (or the majority) of haplotypes of a gene or a region of LD. A number of studies have been conducted to assess the efficacy of htSNPs in covering most of the genetic variation [135] and have shown that these htSNPs can capture at least 80% of the genetic variation within a population [136].

1.5 Aims and Objectives

This study involves the investigation into the impact of inherited variation in candidate cytokine genes playing a key role in the immune system, particularly with reference to cancer - specifically Interleukin- 18 and its receptor complex. Given the pivotal role of Interleukin- 18 in innate and adaptive immunity [137], we hypothesized that polymorphisms in the *IL18RAP* and *IL18* promoter genes may influence susceptibility to oesophageal disease by altering the activities of these proteins.

Bile acid reflux is responsible for the increased incidence and causation of OAC and bile acid receptors have been shown to regulate the metabolism of bile acids-we aimed to characterize the relationship between variation in genes encoding bile acid receptors and GORD leading to OAC. Variants in three genes encoding major bile acid receptors (*FXR*, *PXR* and *RXR*)were investigated.

Another aim was to investigate whether a link exists between genes regulating glucose metabolism and OAC,. A single missense polymorphism in the *TRB-3* gene was examined which has recently been implicated in the pathogenesis of diabetes, insulin metabolism and cardiovascular diseases.

The polymorphisms were chosen to represent the maximal variation of the gene either based on their ability to alter the function of the protein, location of the SNPs or haplotype tagging SNPs (htSNPs), or on the basis that they had previously been associated with disease.

Chapter 2
Materials and Methods

2.1 FINBAR Consortium (Genetic Association studies)

2.1.1 Study Design

As discussed in the previous section, a case-control study was designed to assess the relationship between exposure and the outcome event. The main focus of interest was the presence or absence of the genotypes or haplotypes determined by the chosen set of genes. The outcome events in this study were the factors predisposing to susceptibility and influencing the development of pre-malignant Barrett's and its relationship to oesophageal adenocarcinoma. The objective was to compare exposure history of the associated genotypes between the case and the control groups.

2.1.1.1 Study Subjects and Recruitment

The FINBAR (Factors influencing the Barrett's adenocarcinoma relationship) study commenced in Ireland in March 2002 in collaboration with international researchers and continued until December 2004. The study methods have been described in detail elsewhere[138]. The patients recruited were white Caucasians, aged 35-85 years, who were divided into three: (a) patients with oesophageal adenocarcinoma, (b) patients with long-segment Barrett's oesophagus, and (c) normal population controls, all recruited from both Northern Ireland and the Republic of Ireland. From September 2004 to July 2005, a group of reflux oesophagitis patients were recruited from Northern Ireland only.

Oesophageal adenocarcinoma cases (aged $\leq$ 85 years) were required to have a histologic confirmation of adenocarcinoma within the oesophagus. In situ cancers were not included. Electronic pathology records were used in some cases from Northern Ireland. Cases in the Republic of Ireland were identified from the main

hospitals involved in the diagnosis and treatment of oesophageal cancer. This included St. James's Hospital (Dublin, Ireland) which is a national referral centre for oesophageal cancer. The National Cancer Registry in Ireland based in Cork was also used to recruit patients.

For Barrett's oesophagus, the inclusion criteria were presence of $\geq$ 3 cm of typical Barrett's mucosa seen at endoscopy and the confirmation of presence of SIM by histological examination of biopsy specimens. The electronic pathology records from all pathology laboratories were checked for the histopathlogical diagnosis. Patients with Barrett's dysplasia were excluded. Incident and prevalent cases were included. The length of segment of Barrett's oesophagus was noted and confirmed by reviewing the endoscopy note. In Northern Ireland, pathology reports and endoscopy note review were used to identify cases of Barrett's oesophagus and to confirm the length of the segment of Barrett's oesophagus. In the Republic of Ireland, clinicians from large teaching hospitals in the Dublin and Cork areas contributed by sending details of eligible BO patients to the research personnel.

Reflux oesophagitis group included patients diagnosed in Northern Ireland with macroscopically visible erosive oesophagitis at upper gastrointestinal endoscopy. Erosive oesophagitis was defined as evidence of mucosal breaks or erosions within the oesophagus (grades 2-4 in the Savary Miller/Hetzel- Dent classification or grades B, C, or D in the Los Angeles classification were included). The exclusion criteria were macroscopically visible or histologic evidence of Barrett's oesophagus and/or infection such as candidiasis, also patients with documented dysmotility or gastric outlet obstruction were not included.

Eligible control subjects were adults without a history of oesophageal or other GI cancer or a known diagnosis of BO. In Northern Ireland, controls were selected at

random from the General Practice Master Index (a province-wide database of all persons registered with a General Practitioner) and frequency matched by sex and 5-year age band to the distribution of oesophageal adenocarinoma patients. In the Republic of Ireland, controls were frequency matched (within 5-year age and sex strata) to the distribution of oesophageal adenocarcinoma patients, with a maximum age of 85 years. To include even urban/rural distribution of OAC in the Republic of Ireland, these controls were recruited at random from four general practices (two urban and two rural) in the Dublin and Cork areas. An upper GI endoscopy was not required for control subjects to exclude BO prior to entry into the study as only patients with symptomatic Barrett's oeophagus were included in the study. Less than 5% of the general population have asymptomatic Barrett's oesophagus. The super-normal control population (i.e those selected as controls on the basis of clinical selection) did not have gastro-oesphageal reflux (GOR) symptoms as per clinical data and questionnaire. The random Irish population genetic controls provided by the TCD Biobank were not clinically assessed.

2.1.1.2 Clinical Data Collection

Participants underwent a structured interview with trained interviewers after giving informed written consent. Information obtained included data on symptoms of gastro-oesophageal reflux (questions based on a translation of those used by Lagergren et al. in their Swedish case-control study [54]), height and weight at age 21, weight 5 years before the interview, maximum and minimum weight during adulthood, education, smoking history, occupation and alcohol consumption. Anthropometric measures (height, weight, waist and hip circumference) were taken at the time of interview.

Frequent gastro-oesophageal reflux was defined as symptoms of heartburn and/or acid reflux occurring more than 50 times per year (at least once per week), more than 5 years prior to the interview. Nocturnal gastro-oesophageal reflux symptoms were classified as frequent gastro-oesophageal reflux, which prevented subjects from going to sleep or awoke them from sleep. The reflux symptom score used by Lagergren *et al.*[54] was applied to the FINBAR dataset but scores 1-4 were combined in the analyses because of the small number of subjects in the first 2 categories of the symptom score. Current BMI and BMI 5 years before the interview date were calculated by dividing weight in kilograms (current measured and 5-year self-reported, respectively) by current height in metres squared. BMI at age 21 was calculated by dividing self-reported weight in kilograms at age 21 by self-reported height in metres at age 21 squared.

Current smoking status was defined as having smoked at least one cigarette per day for 6 months or longer, 5 years before the interview date. Previous smokers were classified as those who had quit smoking more than 5 years prior to the interview date. People in the non-smoker group were those who had either never smoked, or smoked less than 100 cigarettes in their lifetime, or less than one cigarette per day for 6 months or longer. [139].

2.1.1.3 Strengths of the Study

The strengths of this case-control study were:

- Population-based recruitment of cases and controls
- The inclusion of cases from each stage of the oesophageal inflammation reflecting the metaplasia-dysplasia-adenocarcinoma sequence
- High response rates among the RO, BO and OAC cases

- The availability of serum from the vast majority of subjects
- Data on the most relevant confounders
- It was less constrained by the natural frequency of the disease
- It was ideal for the assessment of the chosen disease aetiology

2.1.1.4. Bias considerations

The following types of bias were taken into account while designing this study.

- Recall bias: As most of the data was obtained from pre-recorded patient details and information, this bias did not affect this study.

- Confounding bias: This is an inevitable inherent bias of any case-control study. As OAC patients are usually diagnosed at a later age with co-morbidities, multiple confounding variables can influence the results including progressive senile DNA damage.

- Berkson's Bias: This is an issue in studies where subjects who are both exposed and affected are more likely to be admitted to the hospital. As cases were selected from both hospitals and community, this bias was less likely to affect the study.

- Neyman's bias: This bias results in the distortion of the true prevalence of the study if the study subjects have a very short life expectancy. As FINBAR was a collaborative project and included cases representative of other centres without requiring an exact measure of the prevalence, this bias was not introduced.

- Population stratification: All subjects were of Irish ethnicity.

2.1.1.4 Ethical considerations

The FINBAR study had the approval of the Research Ethics Committee of the Queen's University Belfast, Cork Teaching Hospitals and the Research Ethics Committee Board of St. James's Hospital, Dublin.

In designing this study, consideration was given to the fact that genetic susceptibility biomarkers could impact not only the study subjects but also their families. The disease susceptibility biomarkers obtained from an individual may have the potential for revealing important information about exposures, biological effects of exposures, and association with disease for that individual and his family. Therefore, no medical interpretation of the genotyping result was provided to the patients or any other individual however, it was fully acknowledged that study subjects have rights to and can access appropriate information throughout the study period and afterwards.

Steps were taken to ensure the confidentiality of patients and their samples. Written assurance was provided to the Ethical committee that the leftover genetic material would not be used in any other study without fulfilling legal and ethical requirements. The stored genetic and biological material is neither identifiable nor traceable back to the donor. The study subjects were also anonymized after obtaining relevant information and therefore could not be traced back to subject identification. Patients were informed clearly about their right to refuse participation at any stage if they wish. The information leaflet was provided to each participant who was given enough opportunity to carefully read and understand the leaflet. Informed consent form was approved by the ethical committee. Written informed consent was obtained from alive participants. Use of Patients' records/histological records has been explained elsewhere in the material and methods section. Confidentiality of data was ensured by

storing and reporting in a way that a third party could not identify the study participants and access was limited to directly concerned researchers only.

2.1.2 Sample collection and storage

Briefly, 30 ml of blood was drawn in a purple top tube (vacationer) containing EDTA (ethylenediaminetetraacetic acid) as an anticoagulant. All tubes were labelled with patient name, MRN, date of birth and date of sample collection. Blood samples were placed immediately on ice. This was centrifuged and separated within 4 h and buffy coats were stored at -80°C.

2.1.2.1. Quality Control / instrument calibration / validation / reproducibility of results

There is a dedicated room in the institute for the instruments for genetic analysis. All the reagents were as supplied by the manufacturers and used within their date of expiry. The precision of the instruments was checked by repeating the genotype of 20% of whole blood DNA samples by Taqman technology as well as the traditional PCR and restriction digest methods. The results were reproducible in 100% of cases.

2.1.2.2. DNA Extraction

DNA from PET was extracted using PUREGENE DNA purification system (Gentra Systems, Minneapolis, Minnesota, USA). The PET was first dewaxed using xylene and alcohol and 5-10 mg of tissue was used for DNA purification. The PUREGENE system works via alcohol and salt precipitation. The first step is to lyse cells with an anionic detergent in the presence of a DNA stabilizer that inhibits DNase activity, after which RNA is digested. The proteins are digested and removed along with other contaminants by salt precipitation. The DNA is then alcohol precipitated and

dissolved in a DNA stabilizer. This kit provided very consistent results and produced high yields of DNA.

2.1.3 Bioinformatics, Candidate gene selection and Single Nucleotide Polymorphism selection

For genomic region 11q23, the SNP markers used in this study were identified from a previous candidate gene screening study [140].

Single Nucleotide Polymorphisms (SNPs) were selected based either on:

(1) Potential to alter expression (i.e. located in regulatory regions),

(2) Potential to alter the protein coding sequence,

(3) Documented associations of these SNPs with altered expression or activity,

(4) Haplotype tagging.

SNP information was derived from either published studies describing genetic variation with functional or disease association, or from the public databases, HapMap (http://www.hapmap.org/) or Entrez dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). It contains genotypic data for a number of different populations, including those of European ancestry, such as the CEPH population. A technique called haplotype tagging was used to identify which of the SNPs to assay for common haplotypic population variation. The subsets of SNPs that are required to capture the full haplotype information from a larger group of SNPs are called haplotype tagging SNPs, or htSNPs (see section 1.1.2.4).

2.1.4 Genotyping Methods

2.1.4.1 TaqMan Chemistry®

The sensitivity of Taqman genotyping is 96% and the specificity is 98% [141]. Genotyping for all the SNPs was done using TaqMan PCR.

2.1.4.2 Introduction to Taqman technology

TaqMan assay (as described in the Applied Biosystems manual) is also referred to as kinetic PCR or 5' exonuclease based PCR assay. It exploits the 5'-3' exonuclease activity of Taq polymerase (AmpliTaq Gold DNA polymerase). The assay developed by Perkin Elmer Applied Biosystems, Foster City, CA, integrates fluorogenic PCR with a laser-based computerised system. The sequence detection systems used for this study were ABI PRISM 7700 and ABI PRISM 7900HT.

For the allelic discrimination assays two oligonucleotide probes are used, one probe for each allele in the di-allelic SNP. Each probe used in this study had a covalently linked 5' reporter dye and a 3' quencher dye. The reporter used for allele 1 of each SNP was TET (6 carboxy-4,7,2',7'-tetrachloro fluorescein) and for allele 2 the reporter molecule was FAM (6-carboxy fluorescin). The quencher used in all cases was TAMRA (6-carboxy-N,N,N,N'-tetramethyl rhodamine).

2.1.4.3 Assay optimisation

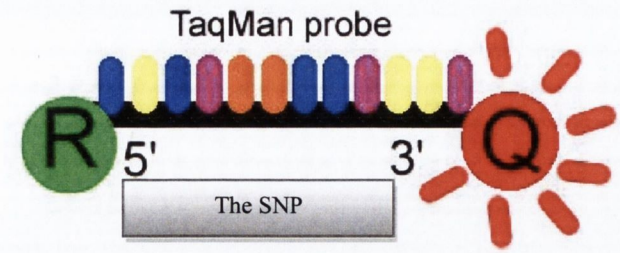
Assays were carried out on two TaqMan sequence detection systems (SDS) as described earlier. The ABI PRISM SDS 7700 took 96 well plates and the ABI PRISM SDS 7900 HT took 384 well plates. Different reaction volumes were used in the two thermocyclers and assays had to be optimized for both the machines and for all the SNPs.

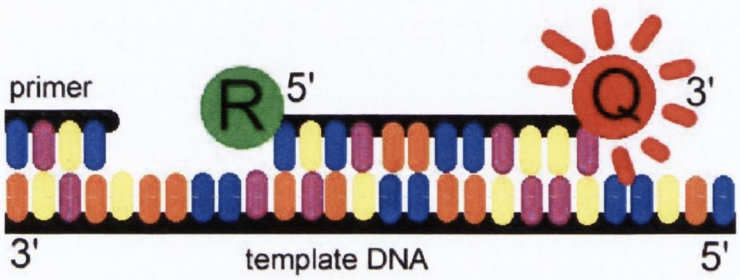
Each plate included 6 "No Template Controls" (NTCs). In case of NTC, sterile water was used in place of DNA to ensure no amplification. The concentrations of both the

primers and the probes are optimized in the first step of the assay and optimisation of the thermocycling takes place in the second step.

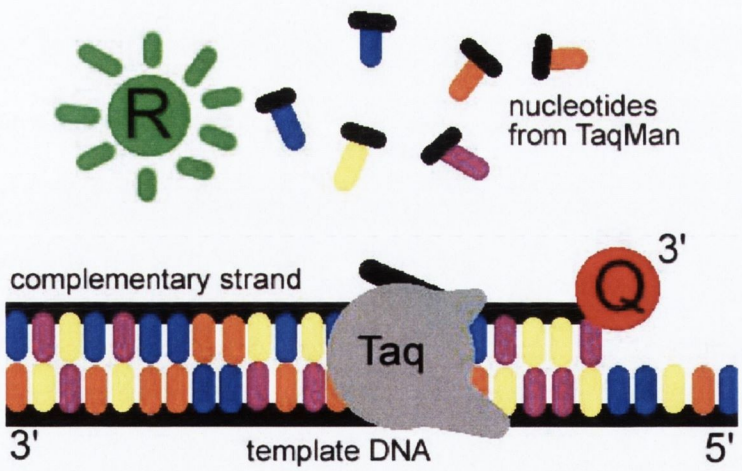
Taqman PCR

In Taqman allelic discrimination assay, two probes are used. Each is specific for one SNP at the bi-allelic locus.





Perfect complimentarity. The probe is tightly bound to template strand



The SNP specific probe is cleaved in the extension process due to the 5′ to 3′ exonuclease activity of the AmpliTaq DNA polymerase. The reporter molecule is separated from the quencher molecule and a signal is generated

Figure 4: The Taqman Assay. The probe consists of two types of fluorophores, which are the fluorescent parts of reporter proteins (Green Fluorescent Protein (GFP) has an often-used fluorophore). While the probe is attached or unattached to the template

DNA and before the polymerase acts, the **quencher (Q)** fluorophore reduces the fluorescence from the **reporter (R)** fluorophore (usually a short-wavelength coloured dye, such as green). (www.probes.com 2003).

2.1.4.4 Probe optimisation

The purpose of probe optimization is to balance the emission of signal from the TET and FAM fluorophores. Thermocycling is not required. The probes were reconstituted to a final concentration of 5 μM. The probes were stored in darkness (containers wrapped in aluminium foil) at -20°C. For optimization, TET labelled probes were kept at a concentration of 200 nM, while the concentration of FAM labelled probe ranges from 50 to 200 nM.

2.1.5 Statistical Analysis

2.1.5.1 Sample size Calculations

Power calculation was done using Genetic power calculator [142]. The power of this study was explored by specifying the following parameters: a disease allele frequency of 5%, disease prevalence of 1%, required level of statistical significance of 0.05, r^2 of between 0.7-1.0 and varying genotype relative risks ranging from 1.3 to 2.0. We also calculated the number of subjects required to give 80% power to successfully detect an association ($p<0.05$).

2.1.5.2 GENEPOP

Genepop [143] (version 3.4 http://wbiomed.curtin.edu.au/ genepop/) was used to test if the distribution of genotypes was in Hardy-Weinberg equilibrium (HWE). All the genotypes in the cases and control groups were tested. Genepop computes exact test for HWE for population differentiation and for genotypic disequilibrium among

pairs of loci using either the complete enumeration method [144] (for loci less than five alleles) or a Monte-Carlo-Markov-Chain (MCMC) method [145, 146]. With the complete enumeration method, an exact p value without any standard error measurement is calculated. A Markov chain method to estimate without bias the exact p value, can also be selected following the algorithm of Guo and Thompson (1992). Genepop also estimates several parameters and correlations such as allele frequencies, allele based statistics for microsatellites.

2.1.5.3 Genetic case-control differentiation analysis of allele, genotype and carrier status frequencies

Allele frequency differences between populations were tested for significance using a Monte Carlo Markov Chain chi-square simulation in GENEPOP (Raymond and Rousset, 1995) and by direct counting using a chi-square, or where appropriate, a Fisher's exact test as implemented by HITAGENE software (www.hitagene.com). Differences in genotype distributions between populations and odds ratio trend tests associated with carrier status were tested for significance using HITAGENE and Epi Info [147], which gave equivalent results.

Epi Info is a useful public domain software package designed for public health practitioners and researchers. It allows database construction, and can carry out epidemiological statistics, tables, graphs and maps. Its Statcalc function computes chi-squares, odds ratios, trend tests, and power calculations.

HITAGENE is web-based software of high quality genetic analysis tools. It is mainly used for population genetics studies designed for genetic studies of human diseases, as a data management system and a variety of high performance analysis programs. Hardy-Weinberg Equilibrium (HWE) testing, haplotype frequency estimation and

linkage disequilibrium testing can be performed using HITAGENE. HWE predicts the frequencies of genotypes expected to be found in randomly assorting population given the allele frequencies present in that population. Deviations from HWE can be caused by significant rates of mutation, migration and the effects of natural selection. In terms of genetic testing, deviations from HWE are often caused by bias or inefficiency in the assay, causing some genotypes to be misrepresented in terms of frequency, and thus this is used as a standard quality control measure.

HWE value of <0.05 indicates significant deviation from the equilibrium.

2.1.5.4. Haplotype Differentiation analysis

Haplotype frequency estimations (HFE) using an expectation maximisation (EM) algorithm were generated on HITAGENE and Haplostats [148]. The EM algorithm considers all possible assignments of haplotype pairs to each unphased genotype, weighted by their relative frequency assuming Hardy-Weinberg equilibrium, and evaluates the haplotype distribution that optimises the probability of the observed data. It is an iterative tool used to calculate maximum-likelihood estimates of haplotype frequencies from multi-locus genotype data.

2.1.5.5 Interaction analysis

Interactions between the polymorphisms in the different genes were identified using crosstabulation analysis performed on SPSS16 and PLINK [149] (SPSS Inc., Chicago IL). Disease status was used as the dependent variable and two SNP loci at a time were used as independent variables. For each SNP, the more frequent allele in the control population was designated as wildtype. Under the additive model, subjects with two wildtype alleles were coded as 0, those with one wildtype and one mutant allele were coded as 1, and those with two mutant alleles were coded as 2. Under the

dominant model, subjects with two wildtype alleles were coded as 0 and those with one or two mutant alleles were coded as 1. Under the recessive model, subjects with zero or one mutant allele were coded as 0 and those with two mutant alleles were coded as 1. The significance of an interaction term was assessed by using the two tailed p-value. Any interaction was then examined by estimating odds ratios with 95% confidence intervals for each of the SNP genotypes/carrier status stratified by the 3 genotypes of the other SNP, also calculated on Epi-info.

2.1.5.6 Linkage Disequilibrium analysis

Linkage disequilibrium (LD) is the non-random association of alleles at two or more loci where the haplotype frequencies in a population deviate from the values they would have if the genes at each locus were combined at random (as described in section 1.4). Presence of LD between two markers can yield useful and complete information about the second marker when the details of the first marker are known. LD in this study was analysed using pairwise r^2 and standard contingency table χ^2-test values as generated by HAPLOSTATS and HITAGENE.

Chapter 3
Genes of the Interleukin-18 pathway and oesophageal disease

3.1 Introduction

3.1.1 Structure and Function of IL-18 and IL18RAP

Interleukin-18 (IL-18) is a member of the IL-1 cytokine family and is produced by a variety of cells, including T and B cells, and a range of antigen presenting cells including activated monocytes, dendritic cells and macrophages [96]. IL-18 stimulates NK cells and T cells promoting primarily a Th1 response, but can also stimulate Th2 and Th17 responses [150, 151]. It induces pro-inflammatory cytokine expression, most notably interferon-gamma (IFN-γ), but also TNF-α, IL-1β, IL-8 among others [152] and promotes cytotoxic effects in T and NK cells by inducing Fas ligand expression[153-156], and perforin activation [157]. The specificity and activity of IL-18 appears to be modified by acting in concert with other cytokines – for example with IL-12 in the production of IFN-γ or IL-23 in the differentiation of Th17 cells.

The IL-18 receptor comprises IL18RAP together with the IL-18 receptor 1 (or alpha) protein (IL-18R1 or IL-1RRP) combined with *IL18RAP* which encodes for the beta-chain of the receptor [158, 159]. IL18RAP forms the signalling chain of this receptor complex and is essential for IL-18 signalling, resulting in the production of IFN-γ [160] in part through activation of NFκB which has been previously shown to contribute to the pathogenesis of oesophageal disease[161]. The IL-18 receptor is expressed by immune (T cells and natural killer cells) [158, 159], fibroblasts, endothelial and epithelial cells[162] including intestinal epithelial cells[163] and Th1 cells in response to IFN-alpha and/or IL12 [164].

Besides its central function in innate and adaptive immunity, IL-18 has been identified both as a promoter and suppressor of oncogenesis. Through activation of an

immune response, and particularly of NK cells, it promotes cell death and tumour regression [98]. In experimental cancer models, *IL18* gene transfection into tumor cells has been shown to enhance both specific and non-specific antitumour immune responses. [99, 100] On the other hand, IL-18 mRNA expression or serum levels have been correlated with development or progression of skin [165] and gastric cancers [166]. Tsuboi et al. [101] showed that IL-18 levels may be associated with biological features including disease severity in patients with oesophageal carcinoma. IL-18 has been shown to upregulate expression of VEGF [167] and thrombospondin-[168] suggesting that it may promote angiogenesis. It is also reported that IL-18 may promote metastasis by inducing cell adhesion molecules [169] and matrix metalloproteinases [170], while promoting immune evasion by increasing expression of fas ligand on tumour cells [171]. Thus the relationship of IL-18 with cancer appears complex and may be tissue and cell type dependent.

3.1.2 Genomic Organization and regulation of *IL18/11q23* region and *IL18RAP/2q11-12*

The human *IL18* (h*IL18*) gene is located on chromosome 11q22.2_q22.3 (111.5 MB), and is composed of six exons and five introns.

Extensive genotype information was available for the *IL18* gene from the IIPGA (Innate Immunity) website (http://innateimmunity.net/PGAs/InnateImmunity/*IL18*/). A total of 38 polymorphic loci were identified in a European population (listed in figure 3.1), out of which htSNPs were selected. HtSNPs rs5744241 and rs187238 are located in the promoter region of the gene and correspond to positions -607 and -137 respectively. The other htSNP rs5744241 is intronic. All remaining SNPs occur in the intronic or untranslated region of the gene.

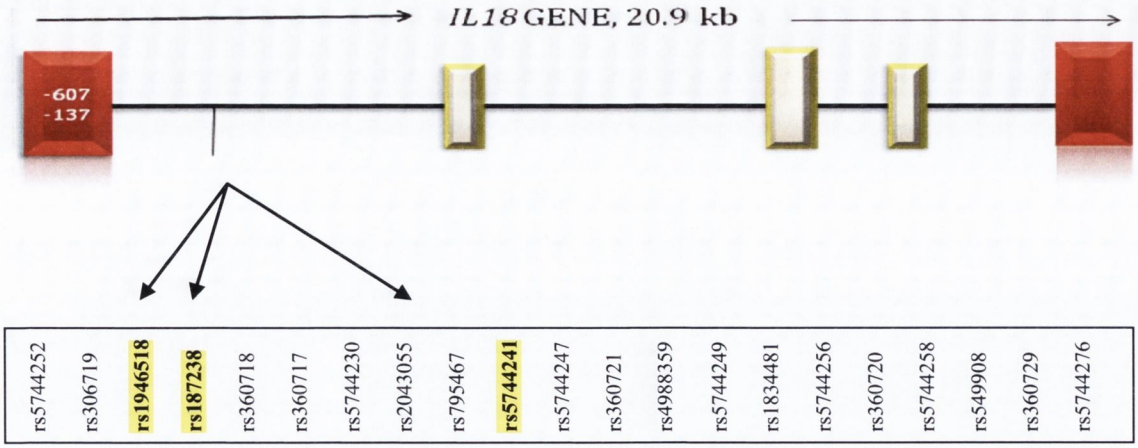


Figure 5: *IL18* haplotype tagging SNP selection. HtSNPs identified, are shaded in yellow.

IL18RAP is contained within a 400 kb LD block that also contains three other genes (*IL-1RL1, IL18R1* and *SLC9A4*). *IL18RAP* locus has been associated with inflammatory bowel disease (IBD) [137], coeliac disease [172-175], type-1 diabetes (T1D) [176] and asthma. [177]

3.1.3 Polymorphisms in the *IL18* and *IL18RAP* gene

IL18 expression is reportedly altered by a number of polymorphisms including three single nucleotide polymorphisms (SNPs) in the *IL18* promoter at positions -137, -607 and -656, relative to the transcriptional start site [178]. The G to C substitution at position -137 abolishes a histone 4 transcription factor-1 (H4TF-1) binding site and the C to A substitution at position -607 disrupts a consensus cyclic adenosine monophosphate (cAMP)-responsive element protein-binding site. Transcription analysis showed that these two SNPs cause altered transcription factor binding and gene expression [178]. Furthermore, polymorphisms of the *IL18* gene have recently been implicated in the susceptibility to a range of inflammation –associated diseases, including atopic asthma, Behcet's disease, cardiovascular disease, and rheumatoid

arthritis [179-181]. In cancer, SNPs in the *IL18* gene (-607 and -137) have been associated with progression of nasopharyngeal carcinoma [182] and ovarian cancer [183].

Similarly, *IL18RAP* (Interleukin-18 Receptor Accessory Protein) locus rs917997 is associated with significantly altered expression of IL18RAP [173] and is strongly associated with coeliac disease, IBD and T1D. Koskinen et al. showed that a risk locus on chromosome 2q11-q12, harbouring *IL18RAP* was associated with coeliac disease. They tested for genetic association for the risk variants at the *IL18RAP* locus in the Finnish, Hungarian and Italian populations and confirmed it with protein expression levels in these populations using western blotting and immunohistochemical analysis[174]. Based on these findings, this locus is relevant for the investigation of oesophageal disease association.

3.1.4 11q23/2 region and oesophageal disease/ Immunology of oesophageal disease re-visited

Serum levels of IL-18 have been shown to correspond to disease severity in OAC. Serum IL-12 and IL-18 amounts as measured by ELISA detected in the blood of oesphageal cancer patients were significantly higher in comparison to control group. Statistically significant differences were found in concentrations of IL-18 according to clinico-pathological parameters like stage of disease, depth of tumour and lymph node metastasis[101] . No studies, to date, have examined the association between genetic polymorphisms in *IL18* genes and oesophageal disease especially OAC and/or Barrett's oesophagus. Recently, polymorphisms in the *IL18* promoter region have been shown to be associated with oesophageal squamous cell carcinoma in the Chinese population. [184]

3.2 Materials and Methods

3.2.1 Study design

A population based case-control study was carried out to assess the association of *IL18* gene with oesophageal disease. The patients were recruited as part of FINBAR study, details of which have been described earlier in chapter 2. SNPs in the *IL18* and *IL18RAP* were selected based on their ability to tag the particular haplotype. HtSNPs rs5744241 and rs187238 are located in the promoter region of the *IL18* gene and correspond to positions -607 and -137 respectively. For the *IL18RAP* locus, the polymorphism rs917997 has been identified as the disease susceptibility locus in previous studies [137, 173, 174, 176].

3.2.2 Genotyping methods

TaqMan genotyping assays were used to perform the genotyping as described earlier in chapter 2.

3.3 Results

3.3.1 Subject Characteristics

The characteristics of each group of subjects are shown in Table 2. Total number of subjects were 1919 (Controls=1238, Reflux oesophagitis=230, Barrett's oesopahgus=224 and OAC=227). There were significant differences in body mass index, years of education, manual/non-manual occupation, gastro-oesophageal reflux (GOR) symptoms and smoking between cases and controls, and significant differences in manual/non-manual occupation and GOR symptoms between BO and controls.

Random Irish controls were also included in the study (n=977) who were recruited from Trinity Biobank Control population for genome wide association studies.

, The Biobank controls (BBC) mean age was 46.2 and male: female ratio was 67.1: 32.9 (data shown in table 2).

Data for haplotype analysis was adjusted for age and sex for all populations using Haplostat [148]. The software possesses inbuilt equations for such adjustments and calculates adjusted haplotype scores according to the type of variable analyzed (binomial, continuous etc).

Table 2: Clinical characteristics of patients

Characteristics	Controls/BBC	ROcases	*P* value RO *vs* Controls	BO cases	*P* value BO *vs* Controls	OAC cases	*P* value OAC *vs* Controls
Age (years) Mean	63/46.2	61.7	0.219	62.4	0.56	64.2	0.26
Sex, number (%)	220(84)/656(67.1)	189(82.2)	0.468	185(83)	0.54	192 (83)	0.99
Male	40 (16)/321(32.9)	41 (17.8)		39 (17)		35 (17)	
Female							
BMI (kg/m^2) Mean	27.2	27.8	0.047	26.9	0.46	28.7	<0.001
Range	19.4-41.9	19.6-43		18.7-42.2		16.7-45.9	
GER symptoms no (%)							
No	211 (81)	140 (60.9)	**<0.001**	60 (25)	**<0.001**	117 (52)	**<0.001**
Yes	49 (19)	90 (39.1)		164 (75)		110 (48)	
Smoking status no (%)							
Never	102 (40.2)	109 (48.4)	**0.026**	87 (39.2)	0.4	45 (20.4)	**<0.001**
Previous	107 (42.1)	68 (30.2)		85 (38.3)		99 (44.8)	
Current	45 (17.7)	48 (213)		50 (22.5)		77 (34.8)	
Education (y)	12	10.8	<0.001	11.3	0.013	10.7	<0.001
Job type, *n* (%)	119(48)	107(48.2)	0.709	130(59.1)	0.016	128(59.5)	0.013
Manual	129(52)	115(51.8)		90(40.9)		87(40.5)	
Nonmanual							
Alcohol, *n* (%)							
Never	69(26.5)	57(26)	0.151	57(25.6)	0.135	65(28.9)	0.004
Ever	191(73.5)	162(74)		166(74.4)		160(71.1)	
Mean(g/d)	26.1	22	0.151	22.3	0.214	19.2	0.012

3.3.2 Genotype and Allele frequencies for all populations

3.3.2.1 Hardy-Weinberg Equilibrium

The genotype distributions of all polymorphisms among the controls and the cases are shown in Table 3 for Hardy-Weinberg equilibrium. As explained in section 2.1.5.3., a significant deviation from HWE is represented by ($p<0.05$), as is seen in Table 3 for *IL18*rs5744241.

Table 3: Hardy-Weinberg Equilibrium for loci in all populations. Figures shown are p values.

	BBC	FINBAR Controls	RO	BO	OAC
***IL18*-137**	0.34	0.73	1	0.697	0.85
***IL18*-607**	0.73	0.88	0.87	0.08	0.01
***IL18*rs5744241**	1	<0.0001	<0.0001	<0.0001	<0.0001
IL18RAP		0.4	0.69	0.61	0.1

3.3.2.2 Genotype and Allele frequencies of IL18RAP SNP locus rs917997

There were highly significant differences in the genotype and allele frequencies of *IL18RAP* (Interleukin-18 Receptor Accessory Protein) locus rs917997 between OAC and control groups. CC genotype for this locus were inversely related with the risk of OAC, conferring a significant protective effect (OR 0.47, 95% CI 0.34-0.65, $p=2x10^{-6}$, p value for C allele = $8x10^{-7}$, and for BO (OR 0.58, CI 0.42-0.79, $p=5x10^{-4}$, p value for C allele=10^{-4}) respectively. The genotype and allele frequencies and carrier status of the homozygote for the disease associated allele compared with alternative alleles are shown in Table 4.

3.3.2.3 Genotype and Allele frequencies of IL18 promoter

There were marginally significant differences in the genotype and allele frequencies of the *IL18* promoter –607 C/A polymorphism between Barrett's oesophagus and the control groups. The –607 CC genotype was associated with a significantly increased risk of Barrett's oesophagus compared with the –607 CA and AA genotypes (OR 1.48, 95% CI, 1.08-2.04, p=0.01). The -607 C allele was associated with a significantly increased risk of Barrett's oesophagus compared with the -607 A allele (OR 1.27, 95% CI, 1.01-1.6, p=0.04).

The OAC cohort of patients showed a similar pattern to Barrett's for the -607 locus with the CC genotype conferring an increased risk in a recessive model of inheritance, but that effect did not reach statistical significance (OR 1.34, CI 0.97-1.85, p=0.06). There were no significant differences between the reflux oesophagitis group and controls for any locus.

3.3.2.4 Genotype frequencies of double homozygotes for both loci at IL18 promoter in Barrett's oesophagus

Although there were no significant differences between cases and controls for the -137 G/C locus, CC homozygosity at -607 and GG at -137 loci conferred an increased risk of Barrett's oesophagus (OR 1.56, 95% CI, 1.12-2.17, p=0.004) over what is seen for the -607 locus alone. Thus the -137 and -607 loci exhibit what appears to be an additive effect in enhancing susceptibility to BO.

Table 4: The genotype and allele frequencies of *IL18* and *IL18RAP* promoter polymorphisms of cases (OAC, BO- Barrett's Oesophagus, RO – Reflux Oesophagitis) and controls. Note that the ORs are similar for BO and OAC versus controls and refluxers. Odds Ratios for genotype comparisons are for the *IL18*-607CC, *IL18*-137GG and *IL18RAP* CC genotypes Vs the other two genotypes as appropriate.

Polymorphisms	Controls *n* (%)	Refluxers *n* (%)	OR (95% CI)	p	BO *n* (%)	OR (95% CI)	p	OAC *n* (%)	OR (95% CI)	p
***IL18* -607 rs1946518**										
Genotypes										
CC	384 (35.8)	68 (37.5)	OR	0.66	88 (45.3)	1.48 (1.08-	**0.01**	84 (42.8)	1.34 (0.98-	**0.06**
CA	508 (47.0)	85 (47.0)	(CC)=1.075		77 (39.6)	2.02)		76 (38.7)	1.8)	
AA	178 (20.0)	28 (15.5)	CI(0.77-1.48)		29 (14.9)			36 (18.3)		
Allele										
C	1276 (59.6)	221 (61)		0.60	253 (65)	1.27 (1.01-	**0.038**	244 (62.2)		0.3
A	864 (40.3)	141 (39)	0.94 (0.75-		135 (35)	1.6)		148 (37.7)	1.12 (0.89-	
			1.18)						1.4)	
***IL18* -137 rs187238**										
Genotypes	582 (53.7)	107 (52.2)		0.67	120		**0.06**	106 (55.2)		0.71
GG	414 (38.2)	82 (40.0)	0.93 (0.69-		(59.7)	1.27 (0.93-		72 (37.5)		
GC	86 (7.9)	16 (7.8)	1.26)		70 (34.8)	1.7)		14 (7.3)	1.05(0.77-	
CC					11 (5.5)				1.44)	
Allele	1578 (72)	296 (72.2)		0.8			0.04	284 (74.0)		0.66
C	586 (27)	114 (27.8)	0.96 (0.76-		310			100 (26.0)		
G			1.23)		(77.1)	1.25 (0.97-				
					92 (22.9)	1.61)			1.05 (0.83-	
***IL18* rs5744241**									1.36)	
Genotypes	40 (16.8)	31 (15.7)		0.74			**0.03**	17 (8.5)		**0.01**
AA	164 (69.1)	132 (67)	0.92 (0.53-					145 (72.8)		
GA	33 (13.9)	34 (17.2)	1.58)		19 (9.7)	0.53 (0.29-		37 (18.5)		
GG					138	0.99)			0.46 (0.24-	
Allele	244 (51.4)	194 (49.2)		0.51	(71.1)		0.07	179 (44.9)	0.87)	**0.05**
A	230 (48.5)	200 (50.7)	0.91 (0.69-		37			219		
G			1.21)		(19.07)	0.78 (0.59-		(55.02)		
						1.03)			0.77 (0.58-	
					176				1.02)	
IL18RAP					(45.3)					
rs917997	674 (62)	115 (58.5)		0.33	212		**$5x10^{-4}$**			**$2x10^{-6}$**
Genotypes	375 (34.5)	70 (35.5)	0.85 (0.63-		(54.6)			80 (43.5)		
CC	38 (3.5)	12 (6.0)	1.17)			0.58 (0.42-		89 (48.4)		
CT						0.79)		15 (8.2)	0.47 (0.34-	
TT	1723 (79.3)	300 (76.1)		0.08					0.64)	
Allele	451 (20.7)	94 (23.9)	0.87 (0.65-				**$10x^{-4}$**	249 (67.5)		**$8x10^{-7}$**
C			1.3)		95 (48.7)	0.63 (0.49-		119 (32.5)		
T					85(43.3)	0.80)			0.55 (0.43-	
					15(8.0)				0.70)	
					275 (70.5)					
					115 (29.5)					

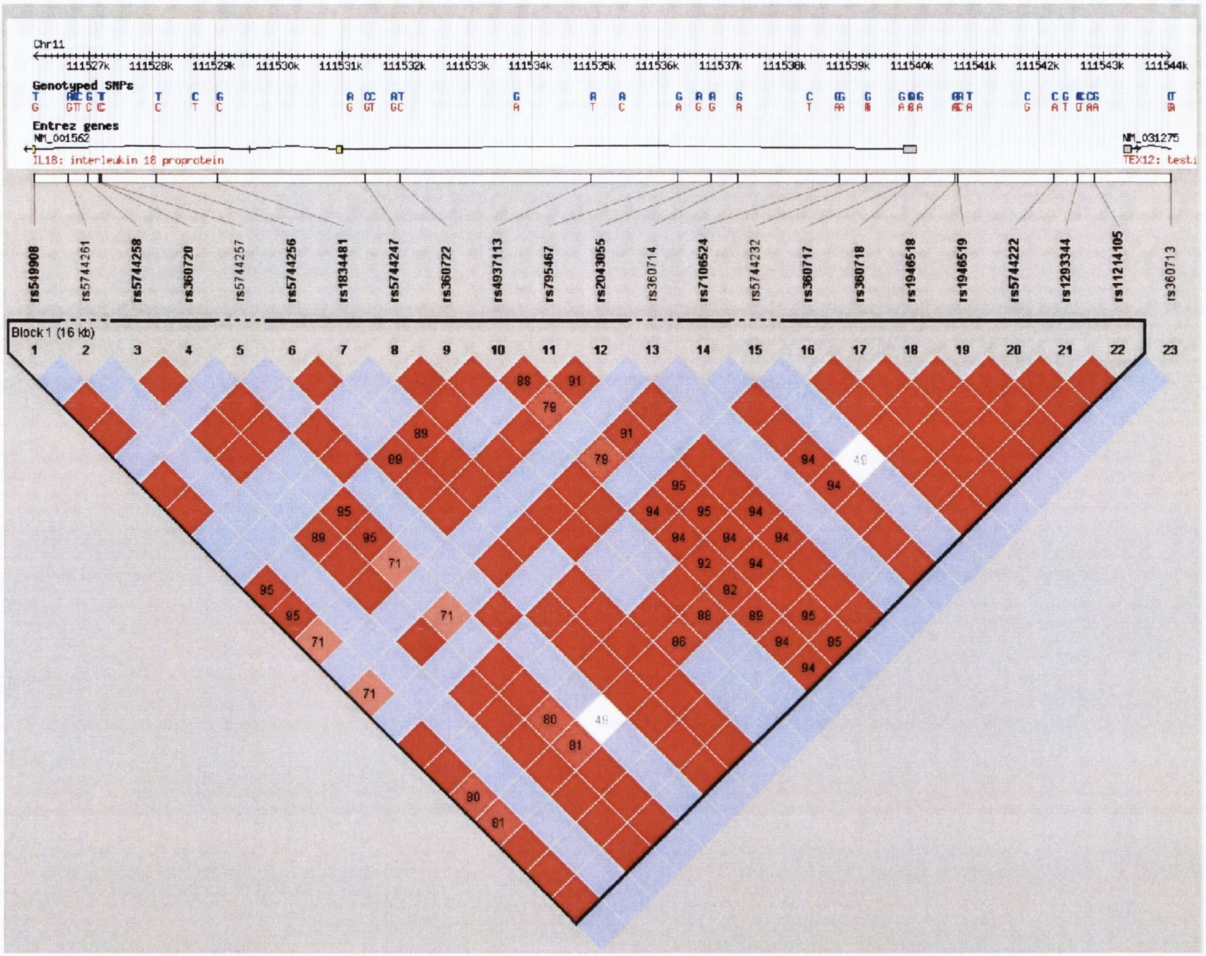


Figure 6: Haploview analysis of linkage between polymorphisms in the IL18 gene. Note that most SNPs are represented in the LD block particularly the promoter region.

3.3.2.5 Genotype and allele frequencies for the locus rs5744241 (IL18 intronic polymorphism)

The AA genotype for locus rs5744241 shows a protective effect for both BO and OAC whereby lack of this genotype was associated with an increased risk for oesophageal disease (OAC, OR 0.46, CI 0.24-0.87, p=0.01), (BO, OR 0.53, CI 0.29-0.99, p=0.03). However as this locus was not in HWE, this finding was ignored.

3.3.2.6 Haplotype frequencies of IL18 promoter polymorphisms

IL18 promoter -137 G/C and -607 C/A polymorphisms show strong linkage disequilibrium ($|D'|$=0.97). We compared haplotype frequencies between Barrett's

oesophagus patients and controls (Table 5). This showed that only three of the four possible haplotypes are present in this population (frequency >1%). *IL18* promoter -137 G/C and -607 C/A polymorphisms are in strong linkage disequilibrium ($|D'|=0.97$). One of the haplotypes, -137G/-607C, was significantly associated with a disease phenotype (BO vs. Controls: 0.65 vs. 0.59; p=0.03). This is the only common haplotype carrying the disease associated -607C allele; the other haplotype carrying this allele is very rare and may be ignored given that these frequencies are computationally derived. Thus the frequency differential observed between groups for the associated haplotypes mirror those observed for the -607 polymorphism, which appears to drive the observed disease association for *IL18*. However, it is interesting that the -137C/-607A and -137G/-607A haplotypes are neutral in terms of disease association, indicating that the -137 locus may have a modifying or secondary effect on the -607 polymorphism, in keeping with the observation that double homozygotes have increased risk of disease.

Table 5: Haplotype frequencies of *IL18* promoter of BE patients and controls and corresponding OR for BE. Total haplotype frequency is the estimated frequency of the haplotype in the BE & Control populations combined. Haplotype frequencies are estimated using the EM algorithm from unphased genotype data using Haploscore software[148]

***IL18* gene promoter haplotypes**	**Total Haplotype frequency**	**Haplotype frequency BO**	**Haplotype frequency Controls**	**Haploscore**	**p**
-137 C/-607 C	0.002	NA	0.0023		
-137 C/-607 A	0.26	0.23	0.26	-1.46	0.14
-137 G/-607 A	0.13	0.11	0.13	-1.13	0.25
-137 G/-607 C	0.60	0.65	0.59	2.144	**0.03**

3.3.2.7 Gene-gene interaction analysis

The gene-gene interaction analysis between *IL18* and *IL18RAP* SNPs was performed using SPSS version 16 and PLINK. High order interactions between polymorphisms of both *IL18* and *IL18RAP* were explored to detect potential combined effects of polymorphisms including both dominant heterozygote and recessive homozygote models. There was no statistically significant interaction between the -607 locus on *IL18* and rs917997 locus of *IL18RAP* gene ($p>0.05$) for Barrett's oesophagus.

3.3.3 Genotype and Allele frequencies for populations pooled together

Our genotype and haplotype analysis of the *IL18* and *IL18RAP* loci between the different oesophageal disease sub-groups (BO and OAC) shows them to be indistinguishable in terms of the allele and genotype frequencies of the *IL18* and *IL18RAP* genes (Fig 8). Similarly, there is no difference between the RO and Controls groups for all polymorphisms. Based on these findings and the clinical observation that OAC develops on a background of BO and thus patients in the OAC group are a subset of BO group, a further analysis was carried out with the various sub-groups of population pooled together as cases (BO and OAC) and controls (Controls and refluxers) in order to maximise statistical power (Table 7). The pooling analysis does increase the multiple testing burden but yields useful information as to the phenotypic correlation of the various genotypes. As BO is a precursor to OAC and develops on a background of reflux oesophagitis, however not all patients with reflux develop BO and/or OAC. Thus characterization of the subjects in two groups is necessary to establish the genotypic differences. The genotype and allele frequency of the *IL18* 607

locus was again significantly, and the *IL18RAP* rs917997 highly significantly, different between pooled cases and controls, the association stronger than in the separate population groups as predicted (OR 1.40, CI 1.10-1.77, p=0.004) for -607 and (CC genotype OR 0.54, CI 0.42-0.68, $p=1x10^{-7}$; C allele, p=0.60, CI(0.50-0.73, $p<1x10^{-7}$) for *IL18RAP*. Odds ratios are not appreciably different when cases are compared with the selected, FINBAR controls on their own, or to the non-selected Irish population controls alone indicating that the pooling of controls and cases lead to skewing of results.

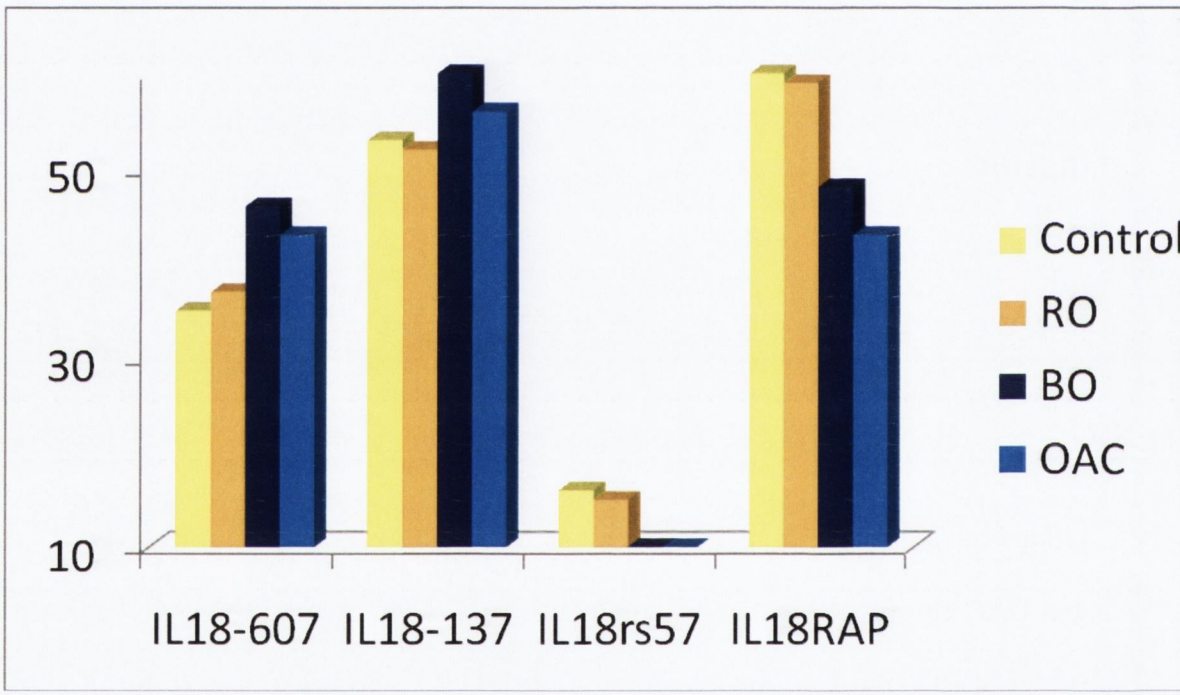


Figure 7: Histogram showing the genotype and allele frequencies among the different groups. Note that the frequencies are similar in Controls and RO while BO and OAC group show similar frequencies to each other as compared with the control/RO group.

3.3.3.1 Hardy-Weinberg Equilibrium

The genotype distributions of all polymorphisms among the controls and the cases are shown in Table 6 for Hardy-Weinberg equilibrium.

Table 6: HWE for all polymorphisms. Figures shown are p values.

	Controls	**Cases**
***IL18*-137**	0.39	0.69
***IL18*-607**	0.64	0.002
***IL18*rs5744241**	<0.00001	<.000001
IL18RAP	0.39	0.19

Table 7: Genotype frequencies across all polymorphisms in *IL18* and *IL18RAP* pooled population. Odds Ratios for genotype comparisons are for the IL18-607CC, IL18-137GG and IL18RAPCC genotypes Vs the other two genotypes as appropriate.

Polymorphisms	Controls n(%)	Cases n(%)	OR	P
IL18 -607 rs1946518				
Genotypes				
CC	452(36.1)	172(44)	1.39(1.10-1.77)	**0.0046**
CA	593(47.4)	153(34.2)		
AA	206(16.4)	65(16.6)		
Allele				
C	1497(59.8)	497(63.7)	1.18(1.00-1.40)	**0.05**
A	1009(40.2)	283(36.3)		
IL18 -137 rs187238				
Genotypes				
GG	689(53.5)	226(57.5)	1.17(0.93-1.49)	0.16
GC	496(38.5)	142(36.1)		
CC	102(7.9)	25(6.3)		
Allele				
C	1874(72.8)	597(75.1)	1.13(0.94-1.37)	**0.18**
G	700(27.1)	197(24.8)		
IL18 rs5744241				
Genotypes				
AA	71(16.6)	36(9.2)	0.76(0.51-1.11)	**0.001**
GA	292(68.3)	281(71.8)		
GG	64(14.9)	74(18.9)		
Allele				
A	434(50.8)	353(45.1)	0.80(0.65-0.97)	**0.02**
G	420(49.2)	429(54.9)		
IL18RAP rs917997				
Genotypes				
CC	789(61.4)	175(46.1)	0.54(0.42-0.68)	**0.0000001**
CT	445(34.6)	174(45.9)		
TT	50(3.8)	30(7.9)		
Allele				
C	2023(78.7)	524(69.1)	0.60(0.50-0.73)	**<0.0000001**
T	545(21.2)	234(30.9)		

3.4 Discussion

To our knowledge, this is the first study to examine functional polymorphisms of the *IL18* gene and its receptor in patients with Barrett's oesophagus and oesophageal adenocarcinoma. This study design is based on analysing candidate functional polymorphisms of the *IL18* signalling pathway in a step-wise biological progression of disease from reflux to cancer. We show that the polymorphism rs917997, which is linked to altered expression of IL18RAP, is strongly associated with both BO and OAC, at levels that approach genome wide significance and surpass this when cases and controls are consolidated. SNPs in *IL18* are weakly associated with Barrett's Oesophagus and OAC prior to correction for multiple testing. This points to the -607 promoter polymorphism as the most likely disease susceptibility locus in *IL18*, the C allele of which has previously been linked to upregulated *IL18* mRNA expression [178]. Here, the *IL18* –607C allele was significantly associated with the risk of Barrett's oesophagus and adenocarcinoma. The –607CC genotype was also significantly associated with the risk of Barrett's oesophagus and the –137G / –607C haplotype conferred an increased susceptibility to Barrett's oesophagus compared with the -607 allelotype alone and double homozygotes, CC at -607 and GG at -137, also showed increased risk of BO. Thus evidence at various levels supports this association. However following a nominal 10x correction for multiple testing only the genotype association in the analysis of combined case and control groups remains significant. Thus bigger sample sizes will be required to definitively characterise this association. We found no evidence of genetic interaction of the *IL18* and *IL18RAP* loci, suggesting they act independently to confer disease susceptibility.

The rs917997 C allele, which is found at significantly decreased frequency in BO and OAC, is associated with increased expression of *IL18RAP* in PBLs [34]. This association is the same direction as observed in coeliac disease. This finding suggests lower IL-18 signalling activity in BO and OAC patients. As discussed above, the evidence indicates that IL-18 can both promote and prevent cancer and these mechanisms are effective in different cell types. Reduced IL-18 activity has been postulated to cause a weaker immune response against tumour cells thus favouring tumor development. A functional interpretation of the involvement of rs917997 is complicated however, given that a truncated form of IL18RAP has been described, whose expression seems to be related to rs917997 alleleotype [174, 185, 186]. This isoform presumably has altered signalling characteristics to the full length protein, although its activity has not been described. It is interesting to note that the same association with *IL18RAP*, pointing to decreased IL-18 signalling, is seen in celiac disease, although this is characterised by high levels of IFN-γ production and significant IL-18 expression in the coeliac lesion[45]. Other apparently contradictory associations are well documented including with *NOD2/CARD15* in IBD and *PTPN22* in various auto-immune conditions [46].

Thus it is not clear how the *IL18RAP* locus contributes to disease. It is possible that alternative IL-18 signalling end-points exist and variants in the *IL18* receptor may indeed have undiscovered biological consequences [174]. It is also conceivable that, since the two IL-18 receptor chains form a high-affinity heterodimeric complex, any genetic variation leading to quantitative and qualitative modification of the complex might have a functional impact, if, for example, the two chains were not expressed in equal amounts[187]. Another possibility is that rs917997 is in linkage disequilibrium with a polymorphism influencing *IL18RAP* activity, although resequencing found no

evidence of this in coeliac patients[34]. Nonetheless the strong evidence of association of *IL18RAP* and to a lesser extent, *IL18*, two separate components of the *IL18* pathway strongly implicates IL-18 signalling in the pathogenesis of oesophageal inflammation and cancer.

There are several reports of cytokine polymorphisms associated with oesophageal disease. Gough et al.. [188] showed an association between functional SNPs in the *IL1 receptor antagonist* gene and BO, and between the *IL10* gene and BO and OAC. The *IL10-1082* locus is associated with increased expression of IL-10, an anti-inflammatory cytokine. Meanwhile, the *EGF* A61G polymorphism, which is associated with higher levels of circulating EGF, confers an increased risk of developing OAC [189]. Pro-survival genes including COX-2 and NFκB appear to be important in the Barrett's to adenocarcinoma pathway and a previous study of the FINBAR cohort has shown the *COX2* 8473 C allele to be significantly associated with OAC [190]. We have previously reported that NFκB, a transcription factor centrally involvedin inflammation, tumourigenesis, and apoptosis, and activated by IL-18, is sequentially activated from GORD through SIM to adenocarcinoma, and activated NFκB in cancer confers a worse prognosis [110, 191, 192]. Wei *et al.*.[184] reported the link between the *IL18* gene promoter polymorphisms studied here and squamous cell cancer of the oesophagus in a Chinese population. Thus genetic associations demonstrated to date strongly indicate that altered activity of cytokine signalling pathways of both a pro and anti-inflammatory nature may predispose to oesophageal disease. Our results indicate that the association of *IL18* with oesophageal disease is likely to be complex possibly having different effects in different cell types.

There is an urgent need to identify biomarkers of risk in BO. Most patients with BO do not develop OAC, yet surveillance endoscopy with biopsy is recommended for the majority of patients diagnosed with the condition[193-195]. This approach incurs costs and causes considerable patient anxiety, and there is limited evidence of effectiveness[195]. The distinct pattern of association of *IL18* polymorphisms with disease phenotype in this study suggests a critical molecular link that might explain the altered response to refluxate in the susceptible group with this polymorphism. Thus patients with reflux who possess these polymorphisms appear to be at risk of Barrett's and probably adenocarcinoma and it will be of particular interest to investigate potential interactions between these susceptibility factors, however a conclusive demonstration will necessitate far greater study sizes than has heretofore been achieved.

This study is one of the largest population based studies reported to date with nationwide subject recruitment and rigorous characterization of OAC and BO phenotypes. Our analysis shows the allele and genotype distributions of *IL18* and *IL18RAP* are very similar in the BO and OAC groups indicating that the *IL18* pathway promotes disease mechanisms common to both. Combining the two groups considerably increases the power and significance of the findings. Our results support other findings in the field including the observation of *IL18* association in a Chinese patient sample.

In summary, this population-based study utilizing a large DNA collection has shown an association between polymorphisms of *IL18* and particularly *IL18RAP* with BO and OAC and suggests that these could be used as genetic susceptibility markers of

BO and OAC. Further studies should focus on other potentially involved genes sharing similar pathways, which may help shed light on the paradigm of GORD, BO and OAC.

Chapter 4
Association of TRB3 (Q84R) with oesophageal disease

4.1 Introduction

4.1.1 Structure, function and genomic organization of TRB-3

An expanding literature on the various members of tribbles gene family (*Drosophilia* and Mammalian) shows that the proteins regulated by these genes may serve an important regulatory function in modulating the activity of various signalling pathways and transcription factors [125]. The mammalian tribbles (TRB-1, TRB-2 and TRB-3) have been identified as regulators of signal processing systems and physiological processes, including development, together with a potential involvement in diabetes and cancer [126, 127]. TRB-3 is mainly localized in the nucleus and has a highly conserved kinase-like domain[196]. There is considerable interest in TRB-3 for its important role in regulating insulin signalling pathways and mechanisms of insulin resistance as these pathways are major determinants for the pathogenesis of diabetes and cancer including OAC. In oesophageal cancer, the proposed hypothesis for these pathways is that insulin resistance or altered glucose metabolism coupled with metabolic syndrome, obesity and diabetes leads to a systemic immune-inflammatory response which predisposes to malignant change [49, 123] and insulin signalling is central to this association. Supporting this hypothesis is the well-documented epidemiological observation of obesity and raised BMI being an independent risk factor for oesophageal adenocarcinoma [51, 123, 124] and a surprisingly strong association exists among central obesity, higher BMI, and metabolic syndrome. In addition, a substantial proportion of Barrett's patients with metabolic syndrome have fasting hyperglycaemia, fasting hyperinsulinaemia and poor sensitivity to insulin [49].

At a cellular level, Akt, a serine/threonine protein kinase, has been shown as a key constituent of the insulin signalling pathways. Impaired Akt activity is involved in human and animal models of insulin resistance [197-199] and thus, mediators of Akt activity are candidates for insulin resistance [128]. TRB-3 has been shown to interact with Akt [200], in fact several lines of evidence suggest that it is a negative regulator of Akt. As shown in Fig 9, insulin binds to its receptor, insulin receptor substrate 1 (IRS-1) to initiate the insulin signalling alongwith Akt phosphorylation. TRB-3 binds to Akt and negatively regulates the Akt activation, predisposing to insulin resistance.

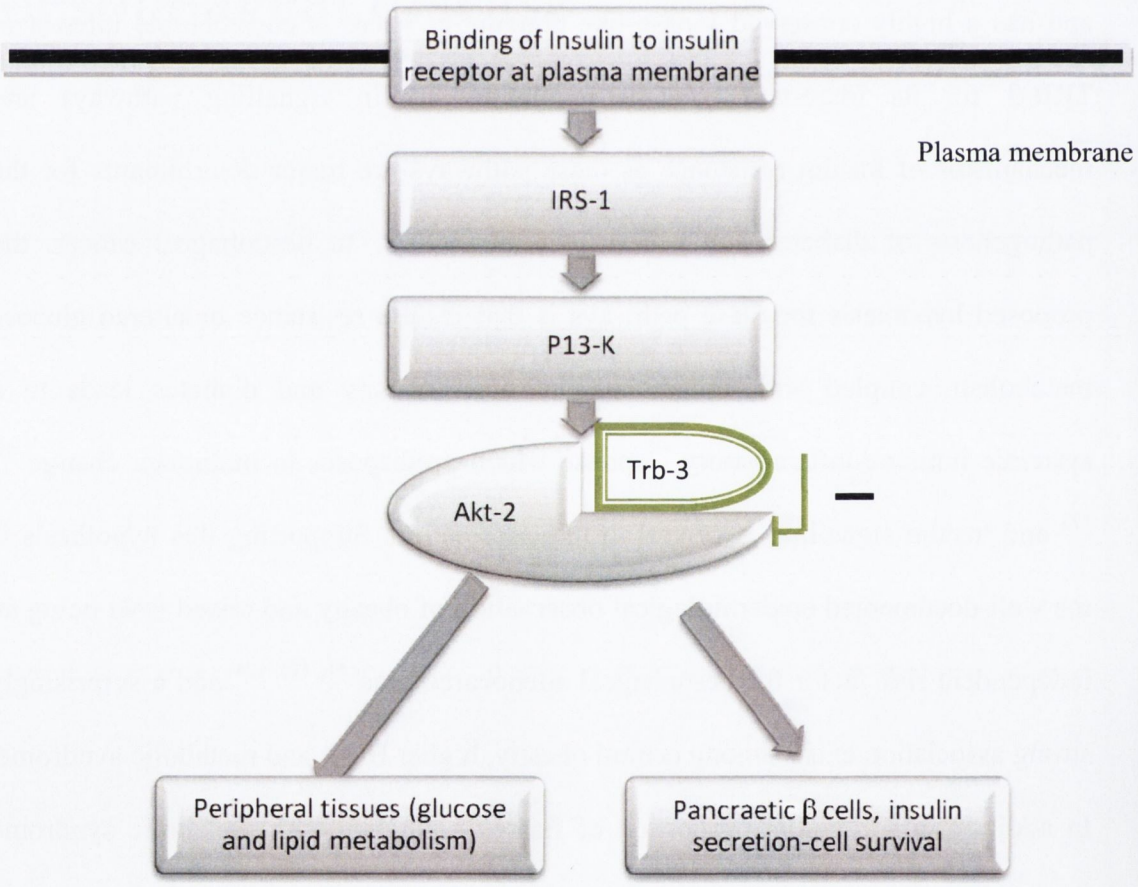


Figure 85: Schematic representation of insulin signalling through the IRS1- PI3K-Akt axis. Insulin binds to its specific tetrameric receptor and stimulates tyrosine phosphorylation of both the insulin receptor b-subunit and of IRS-1 (insulin receptor substrate 1). Then, PI3K (phosphatidylinositol 3,4,5- triphosphate) and Akt (a

serinethreonine kinase, also known as protein kinase B), are activated. TRIB 3 is a negative modulator of insulin signalling among several others and it exerts its inhibitory action by interacting with Akt. Adapted from "The emerging role of TRIB3 as a gene affecting human insulin resistance and related clinical outcomes. Sabrina Prudente, Eleonora Morini, Vincenzo Trischitta,13 Jan 09."

In addition to its role in insulin signalling, TRB-3 also plays important roles in mitosis, apoptosis, cell activation and modulation of gene expression. [125-127] It is known that it regulates of a number of intracellular signalling pathways and interacts with a variety of other cellular proteins including NFkappaB (p65/RelA), Akt/PKB, MKK4 and MKK7, (Fig 10) where TRB-3 negatively regulates their expression. These transcription factors are key determinants in various physiological processes and their main interaction is with the promoter region of TRB-3 [125]. Out of these, NFκB has been strongly implicated in oesophageal disease particularly OAC [110, 161] and hence its interaction with TRB-3 represents a complex regulatory feed-back loop in modulating cellular activities and disease association.

TRB-3 is encoded by the newly identified *TRB3* gene (a mammalian *tribbles* homolog, also known as TRIB3/NIPK, gene ID 57761). As discussed, it has been reported by most [127, 201], although not all [202], studies to affect insulin action by binding to and inhibiting Akt phosphorylation and to play a role in insulin resistance [127, 201]. *TRB3* is located on the 20p13 human chromosome region that has been associated with type 2 diabetes [203, 204]. *TRB3*, therefore, is a candidate gene for insulin resistance [128].

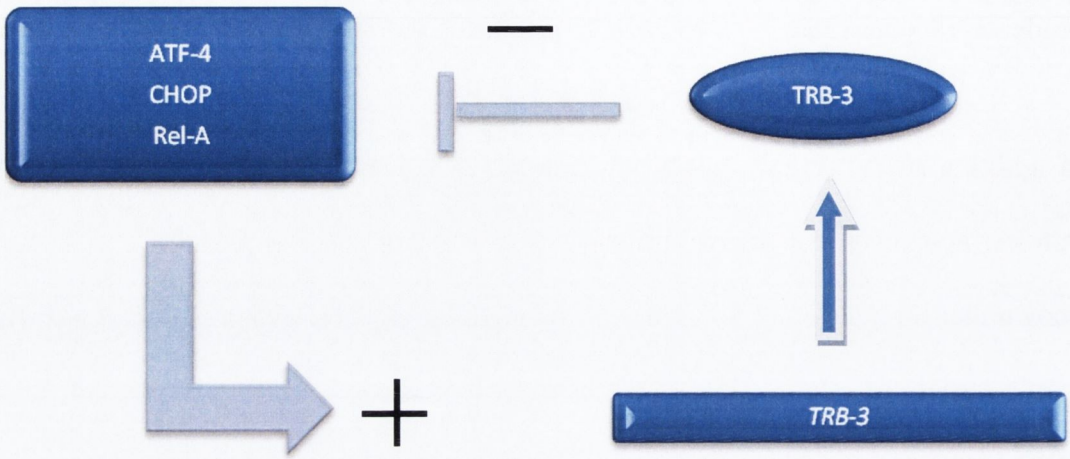


Figure 9: Regulation of expression of tribbles-3 by tribble interacting proteins. (Adapted from "Tribbles: A family of kinase-like proteins with potent signalling regulatory function. Z. Hegedus, A. Czibula, E. Kiss-Toth. Cellular Signalling 19(2007) Pg No. 238-50"

4.1.3. Polymorphisms in the TRB3 gene

A variety of polymorphisms have been identified by resequencing the coding region of TRB3[205] (Table 8). Out of these, five are missense and two are synonymous variants and some of these are available from the National Center for Biotechnology Information (NCBI) database of single nucleotide polymorphisms (dbSNP). Haplotype block construction has showed that Y111Y and A323A, although not situated on a single block, were in partial linkage. The Q84R variant, a missense polymorphism, was found to be both unlinked and prevalent. This polymorphism causes the substitution of a polar uncharged amino acid with a charged one (arginine in place of glutamine at position 84) [205-208]. The rest of the polymorphisms currently under investigation have either very low mean allele frequency (MAF < 5%) or had

little biological relevance. Thus the unlinked Q84R SNP was further tested for association with FINBAR cohort phenotypes.

Table 8: Newly identified or verified variants in the coding region of TRB3 gene. Of the three prevalent ones, A323A and Y111Y were in linkage. Q84R was the unlinked missense SNP selected. Table adapted from "The Functional Q84R Polymorphism of Mammalian *Tribbles* Homolog *TRB3* Is Associated With Insulin Resistance and Related Cardiovascular Risk in Caucasians From Italy, Sabrina Prudente, Marta Letizia Hribal, *Diabetes* 54:2807–2811, 2005".

*Nucleotide positions are based on the NCBI TRB3 mRNA sequence NM-021158. Bold letters indicate minor alleles.

Designation	*dsSNP ID*	*Location*	*Type*	*Codon*	*MAF*
A251**G***	rs2295490	Ex2	Missense	Q84R	15%
T333C	rs6051637	Ex3	Synonymous	Y111Y	35%
G375**A**	rs6084298	Ex3	Synonymous	E125E	Not found
G437**A**	new	Ex3	Missense	S146N	0.5%
G458**A**	new	Ex3	Missense	R153H	1%
T533**C**	new	Ex3	Missense	L178P	0.5%
C541**T**	new	Ex3	Missense	R181C	0.5%
C969**T**	rs6115830	Ex4	Synonymous	A323A	33%
G1026**C**	rs6076497	Ex4	Synonymous	L342L	Not found

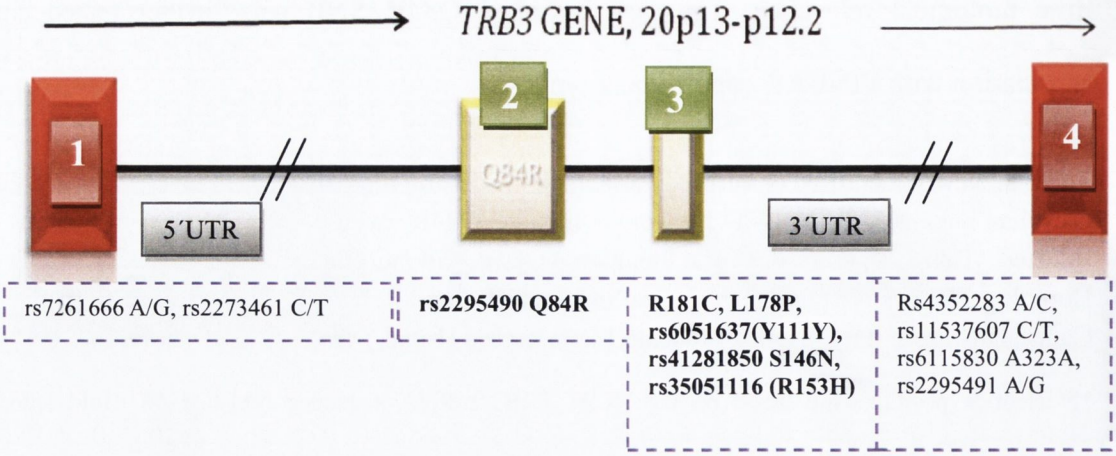


Figure 6: Genomic organization of *TRB3* gene. Coding exons are 2-4 and non-coding is 1, identified in Italian subjects. Missense polymorphisms are indicated in bold. Adapted from "Sabrina Prudente, Eleonora Morini, Vincenzo Trischitta,13 Jan 09."

4.1.4 Hypothesis and Aims

Given the central role of *TRB3* gene in insulin signalling, glucose metabolism and metabolic syndrome, we hypothesized that it is involved in the pathogenesis of OAC and associated with disease particularly metabolic syndrome in our cohort. Metabolic syndrome is best described in association with cardiovascular diseases and diabetes, characterized by increased waist to hip ratio, raised body mass index (BMI), blood pressure and cholesterol and fasting glycaemia. As mentioned, impaired insulin signalling mediated by Akt/TRB-3 interaction is responsible for the development of metabolic syndrome and metabolic syndrome has a significant association with OAC and BO. This hypothesis is further supported by the fact that *TRB3* negatively regulates NFκB, a transcription factor, which has been strongly implicated in the metaplasia-dysplasia-adenocarcinoma sequence in OAC. Hence the aim of this study was to characterize the association of *TRB3* with OAC, coupled with features of metabolic syndrome as manifested by a raised BMI particularly when most patients

with OAC in our cohort had raised BMI as an essential variable in metabolic syndrome.

4.2 Materials and Methods

4.2.1 Study design

A population based case-control study was carried out to assess the association of *TRB3* gene with oesophageal disease. The patients were recruited as part of FINBAR study, details of which have been described earlier in chapter 2. The missense mutation Q84R variant on the gene was selected.

4.2.2 Genotyping methods

TaqMan genotyping assays were used to perform the genotyping as described earlier in chapter 2.

4.3 Results

4.3.1 Patient Characteristics

4.3.1 Gastro-oesophageal reflux

Symptoms of gastro-oesophageal reflux more than 5 years prior to the interview date were strongly associated with Barrett's oesophagus and to a lesser extent with oesophageal adenocarcinoma (Table 9).

Using the symptom scoring system developed by Lagergren *et al.*[54] Barrett's patients were 18 times, and oesophageal adenocarcinoma patients more than 3 times, as likely as controls to have a score in the highest gastro-oesophageal reflux category.

4.3.2 Body Mass Index (BMI)

Body Mass Index (BMI) is an important screening variable in characterizing metabolic syndrome. The associations between BMI (in tertiles) and risk of Barrett's oesophagus and oesophageal adenocarcinoma at the time of interview are displayed in Table 9. The BMI was measured in three time-points (current, at 5 years prior to the interview date or at age 21). This was performed to carefully evaluate the association of BMI with OAC pathogenesis as most OAC patients lose weight after the development of cancer due to dysphagia and other treatment factors. No associations were observed between Barrett's oesophagus and BMI at any stage (current, at 5 years prior to the interview date or at age 21). Current BMI was significantly lower in oesophageal adenocarcinoma patients than in controls, most likely due to cancer-associated weight loss. However, high BMI 5 years prior to the interview date was associated with a more than 2-fold increased risk of oesophageal adenocarcinoma. BMI and body weight was measured as three tertiles to quantify the associated risk, (BMI as <25, 25-28.1 and >28 and weight as <64, 64-70 and >70). OAC patients in the second and third tertile of BMI were more likely to have OAC (OR 1.74 CI 0.66-1.97 for second tertile of BMI and OR 2.69 CI 1.62-4.46 for the third tertile). Oesophageal adenocarcinoma patients were also more likely than controls to be in the highest tertile of BMI at age 21. They also had higher maximum and minimum weights than controls and OAC patients in the third tertile of weight 5 years prior to the interview were more likely to develop OAC (OR 3.22 CI 1.82-5.71 for weight more than 70 kg).

Table 9: Clinical characteristics of the patients. [1]Adjusted for sex, age at interview body mass index (5 yr prior to the interview date), smoking status (never, ex-, current), alcohol intake (grams), years of full-time education and job type (manual, non-manual). 2Symptoms of heartburn and/or reflux more than 50 times per year. [2]Also adjusted for height (centimetres).

	Controls *n*	RE *n*	OR (95% CI)[1]	BO *n*	OR (95% CI)[1]	OAC *n*	OR (95% CI)[1]
Frequent GOR[2]							
No	211(81.2)	140(60.9)	1.00	60(26.8)	1.00	117(51.5)	1.00
Yes	49(18.8)	90(39.1)	2.77(1.80-4.25)	164(73.2)	12.0 (7.64 to 18.7)	110(48.5)	3.48 (2.25 to 5.41)
BMI 5yr ago in tertiles (kg/m^2)	86	57		75		51	
< 25	87	81	1	78	1.00	55	1.00
25-28.1	86	90	1.5(1.01-2.22)	71	0.84 (0.50 to 1.42)	120	1.74 (0.66 to 1.97)
> 28.1			1.58(0.99-2.53)				
Maximum weight in tertiles (kg)[2]	108	88		102	0.85 (0.51 to 1.44)	79	2.69 (1.62 to 4.46)
< 64	81	83	1.00	61	1.00	52	1.00
64-70	70	57	1.22(0.83-1.7)	60	1.00 (0.58 to 1.72)	95	1.20 (0.70 to 2.08)
> 70			1.00		1.25 (0.69 to 2.27)		3.22 (1.82 to 5.71)

4.3.2 Genotype and Allele frequencies

4.3.2.1 Hardy-Weinberg Equilibrium

The genotype distributions of all polymorphisms among the controls and the cases are shown in Table 9 for Hardy-Weinberg equilibrium.

Table 10: Hardy-Weinberg Equilibrium for loci in all populations. Figures shown are p values

	BBC	FINBAR CONTROL	REFLUXERS	BO	OAC
TRB3rs2295490	0.48	0.28	0.80	1	0.57

4.3.2.2 Genotype and Allele frequencies of Q84R polymorphism

The genotype and allele frequencies of *Q84R* (rs2295490*)* polymorphisms among the controls and cases (OAC) are shown in Table 11. The genotype distributions of the polymorphism among the controls and the cases were in Hardy-Weinberg equilibrium.

There were significant differences in the genotype and allele frequencies of the *Q84R* (rs2295490*)* polymorphism between OAC and the control groups. The AA genotype was associated with a significantly increased risk of OAC as compared with the GA and GG genotype (OR 1.49, 95% CI, 1.04-2.14, p=0.02). The A allele was associated with a significantly increased risk of OAC as compared with the G allele (OR 1.41, 95% CI, 1.03-1.94, p=0.02).

Table 12 shows that the control group had significantly more number of patients with normal BMI as compared with the OAC population (61.8% *vs.* 38.2%, OR 1.65, CI 1.06-2.57, p=0.013).

Within the subject group with a higher BMI, the AA genotype was significantly associated with the risk of OAC (OR 2.91, CI 1.16-7.43, p=0.009), as shown in Table 13.

Table 11: The genotype and allele frequencies of *TRB3* locus Q84R (rs2295490) of OAC patients and controls and corresponding OR for OAC

Polymorphisms	Controls *n* (%)	Refluxers *n* (%)	OR (95% CI)	p	BO *n* (%)	OR (95% CI)	p	OAC *n* (%)	OR (95% CI)	p
Q84R (rs2295490) Genotypes										
AA	479 (65.0)	151 (68.6)	1.7 (0.61-5.1)	0.26	124 (62.3)	0.87 (0.37-2.14)	0.48	147 (73.5)	**1.66 (0.59-5)**	0.02
AG	230 (31.2)	64 (29.1)	1.5(0.52-1.6)		67 (33.6)	0.98(0.4-2.4)		48 (24)	1.13(0.39-3.50)	
GG	27 (3.6)	5 (2.2)	1		8 (4.02)	1		5 (2.5)	1	
AA/AG+GG			1.17(0.84-1.64)	0.32		0.89(0.63-1.24)	0.46		**1.49(1.04-2.14)**	**0.02**
Allele										
A	1188 (80.7)	366 (83.1)	1.18 (0.88-1.58)	0.27	315 (79.1)	0.91 (0.68-1.21)	0.53	342 (85.5)	**1.41 (1.03-1.94)**	**0.02**
G	284 (19.3)	74 (16.8)			83 (20.8)			58 (14.5)		

Table 12: The association between BMI and risk of OAC as shown by crosstabulation

	Controls BMI	Obese BMI	Total	OR (p value)
OAC	**47**	**171**	**218**	
% within the BMI group	38.2 %	50.4%	47.2%	
Controls	**76**	**168**	**244**	1.65 CI[1.06-2.57] (p=0.013)
% within the BMI group	61.8%	49.6%	52.8%	
Total	**123**	**339**	**462**	
% within the BMI group	100%	100%	100%	

Table 13: The association of AA genotype for the *TRB3* locus in both OAC and control patients with raised BMI (>25)

	AA genotype TRB3	G Carriers	Total	OR (p value)
OAC	**46**	**9**	**55**	
% within the BMI group	47.4 %	23.7%	40.7%	
Controls	**51**	**29**	**80**	2.91 [1.16-7.43] (p=0.009)
% within the BMI group	52.6%	76.3%	59.3%	
Total	**97**	**38**	**135**	
% within the BMI group	100%	100%	100%	

4.4 Discussion

The incidence of oesophageal adenocarcinoma has increased markedly in the Western world and so is the increased prevalence of obesity. Epidemiological evidence has given an insight into the probable associated factors and obesity characterized as raised BMI and weight is a well-documented strong link in up to 40% of cases[49, 51, 123, 124]. The metabolic syndrome is best described as featuring high BMI, body weight, waist circumference, fasting hyperglycaemia and reduced sensitivity to insulin[209, 210]. These factors are also positively associated with the prevalence and mortality rates from many diseases and cancers [49]. Although the proposed mechanisms of pathogenesis in these diseases are the altered systemic inflammatory state coupled with insulin resistance [49] in subjects with raised BMI and metabolic syndrome, the details and intermediate steps of this key pathway are poorly understood.

In the context of insulin signalling pathways, several inhibitors of insulin signalling have been recently proposed and are currently the focus of attention[211-213]. Among them is the TRIB-3, a mammalian tribbles homolog also known as TRIB3/NIPK (gene ID 57761) which affects insulin signalling by interacting with Akt-2 (Fig-2)[127, 205]. Akt, a serine/threonine protein kinase, is a key mediator of insulin signalling [200] : impaired Akt activity is involved in human and animal models of insulin resistance[197, 199] . TRB3 has been reported by most [127, 201] studies to affect insulin action by binding to and inhibiting Akt phosphorylation and to play a role in insulin resistance. In addition, recent findings have evidenced that TRB3 acts as a potent negative regulator of PPAR-γ, a master modulator of adipocyte differentiation, and tightly controls adipogenesis[214] . Finally, the TRIB3 gene (Fig. 2) is located on

the 20p13 human chromosome region that has been associated with type 2 diabetes[203, 204] . TRIB3 is, therefore, an excellent candidate gene for insulin resistance and related disorders.

A prevalent TRB3 missense single nucleotide polymorphism (SNP) was reported a few years ago (i.e., Q84R, where arginine replaces glutamine at position 84; rs2295490)[205]. This substitution is equivalent to a gain of functional mutation as shown by several studies. Prudente et al. [205] transfected Q84 or R84 variants in HepG2 hepatoma cell lines and found that insulin induced *Ser*473-Akt phosphorylation was reduced significantly in both transfected lines compared with control HepG2 cells but the R84-transfected cells showing a stronger effect than Q84-transfected cells (44% vs. 22% respectively). These data support the biological evidence that the TRB3 R84 variant is a stronger inhibitor of insulin signalling as compared with the ''wild type'' (i.e., more common) Q84 variant and might play, therefore, a role in affecting insulin sensitivity [205]. Similar associations were reported for this variant with impaired insulin mediated nitric oxide (NO) production in human endothelial cells[206] and carotid atherosclerosis[215].

This is the first study to our knowledge to demonstrate the association of the TRB3 gene with oesophageal disease especially oesophageal carcinoma. The Q84R variant, previously shown as a major determinant for modulating in vivo insulin resistance and related clinical outcomes, hereby shows a significant association with oesophageal carcinoma (OR 1.49, 95% CI, 1.04-2.14, p=0.02).

Our findings from this study support the hypothesis linking insulin resistance, metabolic syndrome (particularly BMI) and oesophageal disease. Of note, in our cohort, patients with BO did not have higher current or previous BMI (> 5years ago at the time of interview) as compared with controls and this group did not show any

association with the *TRB3* variant. However, in OAC, a high BMI 5 years prior to the interview date was associated with a 2.5 fold increased risk of oesophageal adenocarcinoma which is similar to reports in other case-control studies [51, 52, 216-218]. Some studies have suggested that a high BMI is associated with an increased risk of Barrett's oesophagus [219-221], although Caygill *et al.* [221] suggested that obesity is only a risk factor for Barrett's oesophagus in young people. One possible mechanism for the association between BMI and oesophageal adenocarcinoma has been suggested to be through the increased production of free insulin-like growth factor-1 in obese subjects, which stimulates cell proliferation and inhibits apoptosis in both BO and OAC [222, 223, 224, 225] and TRB-3 has been shown to interact with IGF-1/Akt signalling pathway [226-228].

In conclusion, this is the first of the FINBAR genetic studies to identify a significant link to the risk of adenocarcinoma with genes involved in insulin resistance and metabolic syndrome. This study, although limited in size, is one of the biggest cohorts covering all three stages of oesophageal disease, and is meticulously documented clinically. Here we show that oesophageal adenocarcinoma is associated with Q84R variant in the *TRB3* gene and differs from controls, refluxers and Barrett's oesophagus by being associated with a higher BMI and weight ratios. These factors could be implicated in the development of oesophageal adenocarcinoma from Barrett's oesophagus. Although the OR of the disease (OAC) associated with this polymorphism is only ~1.5, it represents the first finding of a genetic association between SNPs in genes of the glucose metabolism pathway and oesophageal disease. In order to properly quantify the importance of this observation, it needs to be replicated in other populations and other loci in these pathways should be examined. It is hoped that these findings will help direct future research into the mechanisms

underlying oesophageal adenocarcinoma and the development of prevention strategies with further observational and interventional studies.

Chapter 5
Association of Xenobiotic metabolism (bile acid receptor polymorphisms) with oesophageal disease
PXR, FXR AND RXR

5.1 Introduction

Barrett's oesophagus (BO), defined by the detection of intestinal metaplasia in the oesophagus at histological examination [105], is the most important risk factor for oesophageal adenocarcinoma (OAC) [121]. In subjects with BO the annual incidence of OAC is approximately 0.4–2.1% [229]. Gastrooesophageal reflux disease (GORD) is a known risk factor where the noxious agents responsible for injuring the oesophageal mucosa may originate either from the stomach (hydrochloric acid and pepsin) and or the duodenum (bile acids and pancreatic secretions). Mixed bile and acid reflux is more harmful to the oesophageal mucosa than acid reflux alone in humans [113, 114]. This was proven in an experimental rat model whereby the creation of a duodenooesophageal anastomosis led to oesophagitis, intestinal metaplasia and eventually OAC [230]. However, diversion of biliary contents did not lead to regression of BO, but prevented OAC [231]. The exact mechanisms of bile acid induced injury in malignant transformation of BO remain elusive although several lines of evidence suggest that a loss of apoptotic mechanisms may be responsible[232].

5.1.1 Structure and Function of Bile Acid Receptors (Xenobiotics)

Bile acid receptors are orphan nuclear receptors activated by bile acids, in particular chenodeoxycholic acid, which are abundantly present in bile, a component of refluxate in BO[114, 230]. Binding of these receptors by their ligands leads to their activation as transcription factors and the induction of diverse target genes. With respect to cancer, certain nuclear receptors have been suggested to play a role in the regulation of apoptotic pathways [116], which are of particular relevance in BO[116, 121] and it has been shown that nuclear receptors such as PXR and PPARs can specifically

influence the presence and progression of inflammation. More recent evidence has suggested considerable interaction amongst these xenobiotic receptors between each other and also with other cell signalling, immune and inflammation pathways[117, 233, 234].

5.1.1.1 PXR

The pregnane X receptor (PXR) is a member of the nuclear receptor superfamily that includes the steroid, retinoid and orphan receptors. It is a ligand activated transcription factor that plays as key role in the xenobiotic response system by preventing toxic accumulations of xenobiotics within cells [235-237]. PXR ligands include a broad range of both endogenous and exogenous substances including bile acids, steroids, antibiotics, many prescription drugs and other externally derived compounds. Activated PXR induces the expression of genes involved in the metabolism of xenobiotics, including enzymes involved in their oxidation, conjugation and export from cells [238]. The CYP3A family and the multiple drug resistance gene (*MDR1/ABCB1)* are well studied examples of PXR activated genes. CYP3A4 is a critically important DME (drug metabolizing enzyme); it is estimated that 50% of all prescription drugs are metabolized by CYP3A4, which like MDR1 and PXR, is expressed in tissues which are highly exposed to xenobiotics, including the intestine and liver. The ability of PXR to interact with other proteins/transcription factors is demonstrated by the fact that PXR induction of CYP3A4 is restricted to the liver by an obligate interaction with the liver specific nuclear receptor, hepatocyte nuclear factor-4 alpha (HNF4α) on the CYP3A4 promoter[239]. Thus it may be predicted that the genes activated by PXR in different tissues are highly specific.

5.1.1.2 FXR

The farnesoid X receptor (FXR) is another member of the nuclear receptor superfamily. FXR is known to bind a variety of bile acids and may be the principal receptor which responds to these compounds [116, 240-242]. For example, it is strongly activated by the bile acids chenodeoxycholic acid (CA), deoxycholic acid, and lithocholic acid (LCA) [240, 241], which are found at significant concentrations in the gastric refluxate. Several different isoforms of FXR have been identified (discussed below) with their expression most abundant in the liver, but is also expressed in the gut (particularly the ileum) and the kidney. When activated, FXR forms a heterodimer with RXRα and binds to DNA response elements found upstream of FXR activated genes. [243]. This receptor has been shown to play a critical role in controlling bile acid homeostasis, lipoprotein and glucose metabolism, hepatic regeneration, growth of intestinal bacteria and the response to hepatotoxins [244]. FXR is also an important regulator of intestinal innate immunity, cell signalling and inflammatory response genes including Nuclear Factor kappa B (NFkappaB)[117, 245].

5.1.1.3 RXR

Retinoid X receptor alpha (RXRα) is a nuclear hormone receptor that mediates retinoic acid (RA) dependent transcription. The RXRs are a distinct group of retinoid receptors with unique structure, tissue distribution, ligand specificity and target gene activation[246]. They can function as a homodimer or can heterodimerise, not only with the other retinoic acid responsive receptors (RARs), but also with other nuclear receptors, including PPARα, CAR (NR1I3), PXR (NR1I2), LXRα(NR1H3) and FXR (NR1H4) [118, 247]. PXR heterodimerises with RXRα to form a functional transcription factor, upon which it binds and activates its cognate response elements in the promoters of target genes. Thus, RXRα plays an integral role in regulating many diverse physiological pathways including cholesterol, fatty acid, bile acid, steroid, and

xenobiotic metabolism and homeostasis by being a binding partner for nuclear receptors activated in response to these compounds.

5.1.2 Genomic Organization and regulation of Bile Acid Receptors

5.1.2.1 PXR

The human *PXR* (*PXR/NR1I2*) gene spans 38kb and is located at *3q11- 13*. It consists of nine exons; exons 2 to 8 contain the coding region of 434 amino acids (Figure 12). Multiple number of transcripts, mostly differing in their 5′ UTR sequences and at least three protein isoforms, have been described [248-250].

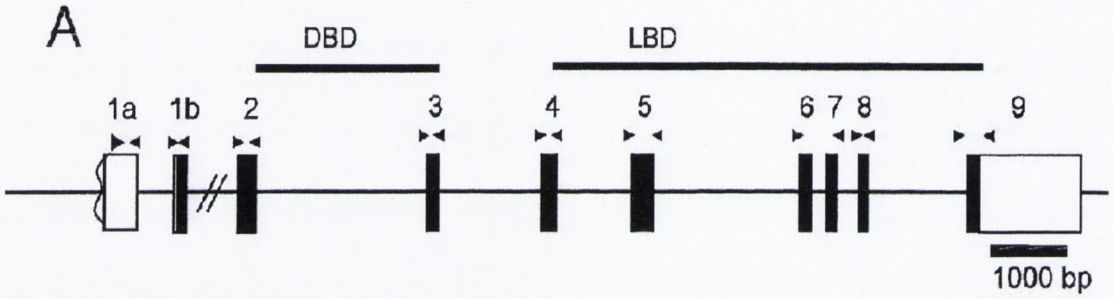


Figure 11: Genomic organization of PXR/ABCB1. Numbers represent exons, protein-coding regions are depicted as filled boxes and the 5' and 3' untranslated regions as white boxes. DBD: DNA binding domain, LBD: Ligand binding domain. (Hustert et al.., 2001)[251]

5.1.2.2 FXR

The *FXR*(*NR1H4*) gene gives rise to a number of different, expressed, splice variants in humans, including FXR-α1, FXR-α2, FXR-β1, and FXR-β2[252]. The α and β forms of FXR are produced from alternative promoters with separate AUG initiation codons. Hence, FXR-β transcripts are 187bp shorter than FXR-α transcripts but generate proteins with an additional 37 amino acids at the amino terminus. FXR-α is highly expressed in the adrenal gland and liver, and to a lesser extent in the duodenum, kidney and small intestine. The FXR-β variants are predominantly expressed in the colon, duodenum and kidney, with lower expression in the kidney.

FXR-α2 and FXR-β2 have a greater binding affinity than FXR-α1 and FXR-β1 for FXR response elements, purportedly due to the presence of an extra four amino-acid residues (MYTG) in the hinge domain derived by differential splicing of exon 5 (Figure 13)[253]. The *FXR* gene is located on chromosome 12q23.1, covering 89.5kb of genomic sequence. It contains 11 exons, with the initiation codon at the 3′ end of exon 3, with exons 1 and 2 and the 5′ half of exon 3 containing the 5′ UTR [252]. An alternative exon 3a encodes the FXR- β variant, which has an alternate N-terminal sequence.

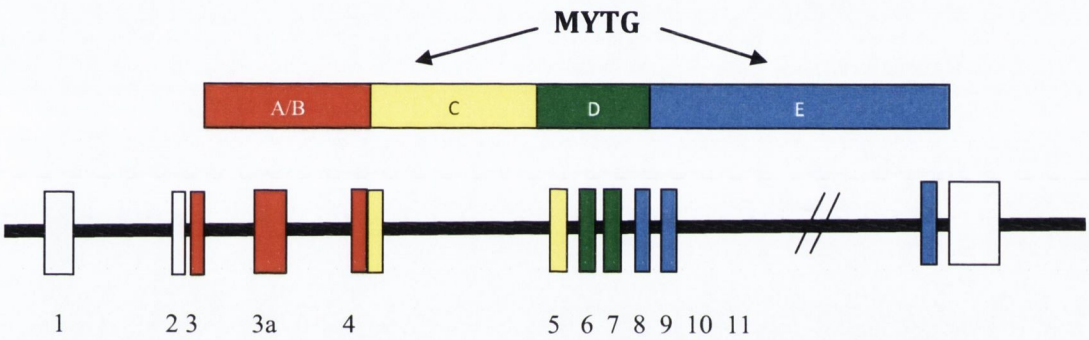


Figure 12: Exon/intron map of the human *NR1H4* locus. The domain structure of the FXR protein is shown on the top with the location of the four amino acid insertion being denoted. The five domains are colourcoded and labelled, with lines and colour-coding denoting the exons encoding each domain. Grey shading represents untranslated regions. Exons are numbered with the novel 5′ exon being denoted as exon 3a. FXR-α2 and FXR-β2 contain an extra 4-amino acid insert, MYTG. (Huber et al., 2002)

5.1.2.3 RXR

Located at 9q34 [254], *RXRα/NR2B1* is 38.95 kb long, with 9 exons (according to Ensembl). Three common SNPs in intron 7, intron 9 and in the 3′ UTR regions have been identified in the Caucasian population [255] for *RXRα/NR2B1*. Hapmap describes 12 polymorphic SNPs in a Caucasian sample.

5.1.3 Polymorphisms in the gene

Zhang et al.. resequenced the *PXR/NR1I2*gene and in so doing, identified a total of 38 SNPs spanning the promoter, intervening sequences, exons and untranslated mRNA regions (UTRs)[256]. The SNP loci -25564 (G/A) (rs12721602) and -25385 (C/T) (rs3814055) are promoter polymorphisms. Importantly, functional studies showed these, and several other SNPs in the *PXR/NR1I2* gene, to be associated with altered regulation of downstream drug and xenobiotic metabolism genes which are regulated by PXR. Of the PXR SNPs included in our study, -25385(rs3814055) has been associated with altered *CYP3A4* regulation, while the -25564 (rs12721602) SNP alters the sequence of potential transcription factor binding sites in the PXR promoter. A previous study in our laboratories showed these *NR1I2* SNPs to be most significantly associated with IBD from a range of SNPs assayed [257].

In the FXR/*NR1H4* we selected four SNPs from 24 that were described on Hapmap based on haplotype tagging with Haploview [257]. These 4 SNPs tag most of the haplotypes reconstructed from the 24 Hapmap SNPs. Thus the four htSNP;*FXR*_SNP1 (A/G) (rs4764980), *FXR*_SNP2 (G/A) (rs7956050), *FXR*_SNP3 (A/G) (rs1030454) and *FXR*_SNP4 (C/T) (rs7304328), were assayed in the cases and control populations. *FXR*_SNP1 is located in the 5′ UTR, and *FXR*_SNP2 to 4 are intronic polymorphisms.

We selected *RXR*a_SNP1 (A/G) (rs3132296), *RXR*a_SNP3 (A/G) (rs4240705) and *RXR*a_SNP5 (G/T) (rs1045570) based on haplotype tagging by SNPtagger, using 12 polymorphic SNPs from Hapmap. *RXR*a_SNP5 is located in the *RXR*α 3′ untranslated region (UTR) while *RXR*a_SNP1 and *RXR*a_SNP3 are intronic polymorphisms.

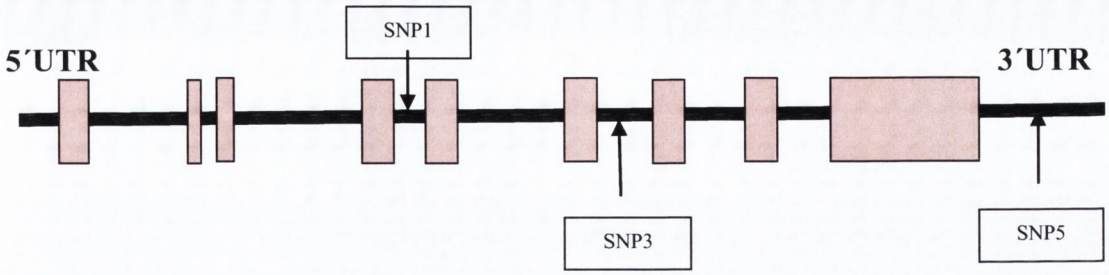


Figure 13: Diagram of *RXRα* gene showing relative locations of selected SNPs (exons in pink).

5.1.4. 11q23/2 region and oesophageal disease/ Genetic xenobiology of oesophageal disease re-visited

Altered xenobiotic metabolism has been linked with various immune related diseases and malignancies including oesophageal disease particularly its pre-malignant precursor Barrett's oesophagus. Increased expression of the xenobiotic receptors (FXR and RXR) as well as CYP3A4 has been reported in oesophageal disease[119-122]. Gene polymorphism studies have looked at some bile acid receptor variations but to date, no research has been conducted on the polymorphisms in the PXR, RXR and FXR genes. Given the suggested importance of bile and bile acid in the pathogenesis of Barrett's adenocarcinoma, the genes encoding these bile acid receptors (PXR, RXR and FXR) are suitable candidates for SNP analysis in the FINBAR cohort.

5.2 Materials and Methods

The materials and methods, and patient and control samples are as described earlier (Chapter 2, section 2.1.1.1. and 2.1.2. Patients were recruited as part of FINBAR study and genotyping performed by Taqman Genotyping assays.

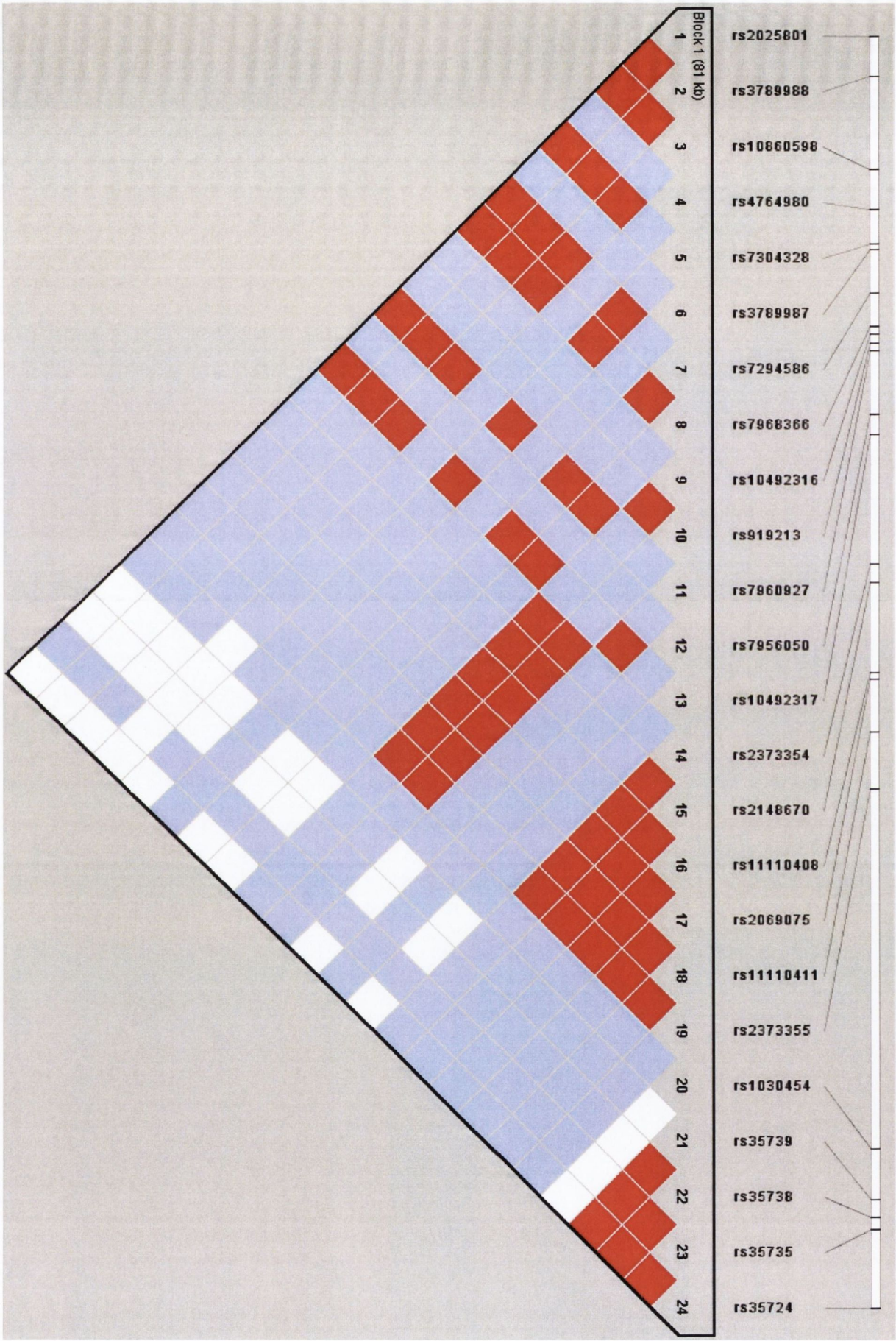
5.3 Results

5.3.1 Clinical Characteristics

Table 14 summarizes the clinical features of the four groups. The RO, BO and OAC groups were more likely to suffer from reflux symptoms, had increased BMI's and were more likely to smoke as compared with reflux-free controls ($p<0.05$).

Table 14: Clinical Characteristics of patients

Characteristics	Controls	RO cases	*P* value RO *vs* Controls	BO cases	*P* value BO *vs* Controls	OAC cases	*P* value OAC *vs* Controls
Age (years)							
Mean	63	61.7	0.219	62.4	0.56	64.2	0.26
Sex, number							
(%)	220 (84)	189 (82.2)	0.468	185 (83)	0.54	192 (83)	0.99
Male	40 (16)	41 (17.8)		39 (17)		35 (17)	
Female							
BM (kg/m^2)	27.2	27.8	**0.047**	26.9	0.46	28.7	**<0.001**
Mean	19.4-41.9	19.6-43		18.7-42.2		16.7-45.9	
Range							
GOR							
symptoms no	211 (81)	140 (60.9)	**<0.001**	60 (25)	**<0.001**	117 (52)	**<0.001**
(%)	49 (19)	90 (39.1)		164 (75)		110 (48)	
No							
Yes							
Smoking	102 (40.2)	109 (48.4)	**0.026**	87 (39.2)	0.4	45 (20.4)	**<0.001**
status no (%)	107 (42.1)	68 (30.2)		85 (38.3)		99 (44.8)	
Never	45 (17.7)	48 (213)		50 (22.5)		77 (34.8)	
Previous							
Current							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rs10492317
rs2373354
rs2148670
rs11110408
rs2069075
rs11110411
rs2373355
rs1030454
rs35739
rs35738
rs35735
rs35724

Figure 14: Linkage disequilibrium analysis of the various polymorphisms in the *FXR* gene

5.3.2 Genotype and Allele frequencies

5.3.2.1 Hardy-Weinberg Equilibrium

The genotype distributions of all polymorphisms among the controls and the cases are shown in Table 15 for Hardy-Weinberg equilibrium.

Table 15:Hardy-Weinberg Equilibrium for loci in all populations. Figures shown are p values

	BBC	FINBAR Controls	RO	BO	OAC
PXR-25385	0.75	1	0.1	0.66	0.3
PXR-25564	0.73	0.66	0.622	0.62	0.80
RXRrs1045570		1	0.1	0.8	0.08
RXRrs3132296	0.08	0.18	0.87	0.86	0.75
RXRrs4240705	0.43	0.49	0.24	0.35	0.36
FXRrs1030454		0.61	0.61	0.58	1
FXRrs7956050	0	1	0	0	0
FXRrs4764980	0.03	0.26	0.77	0.39	0.77
FXRrs7304328	0.22	0.08	0.06	0.01	0.08

5.3.2.2 Genotype and Allele frequencies of bile acid receptor polymorphism

The genotype and allele frequencies were similar for all groups in all the PXR, RXR SNPs and two FXR SNPs, (OR, CI and p values in table II). FXR SNP rs7956050 and

rs4764980 were associated with a significant risk of OAC [GG genotype p=0.03 (OR 2.56, CI 1.06-6.43), G allele p=0.02 OR(2.11, CI 1.07-4.22) and [AA genotype p=0.01 (OR 1.53, CI 1.06-2.20), A allele p=0.02 OR(1.29, CI 1.03-1.62), Table 16.

Table 16: Genotype and corresponding allele frequencies of the polymorphisms chosen for *PXR*, *FXR* and *RXR*. ORs are for the disease causing allele homozygotes compared with the opposite allele carrier status.

Polymorphisms	Controls N(%)	Refluxers N(%)	OR(CI) P	BO	OR(CI) P	OAC N(%)	OR(CI) P
PXR -25385 (rs3814055)							
Genotype							
CC	308(33.7)	87(39.5)	1.28(0.94-1.76)0.1	76(36.5)	1.08(0.88-1.36)0.50	66(34.3)	1.03(0.73-1.45)0.86
TT	163(17.8)	39(17.7)		35(16.8)		39(20.3)	
CT	442(48.4)	94(42.7)		97(46.6)		87(45.3)	
Allele							
C	1058(57.9)	268(60.9)	1.13(0.91-1.41)0.25	249(59.8)	1.08(0.87-1.35)0.47	219(57.0)	0.96(0.77-1.21)0.74
T	768(42.0)	172(39.0)		167(40.1)		165(42.9)	
PXR-25564 (rs12721602)							
Genotype							
GG	781(68.0)	139(68.4)	1.02(0.7-1.43)0.9	136(69.0)	1.05(0.75-1.47)0.7	135(68.1)	1.01(0.72-1.41)0.96
AA	35(3.0)	7(3.4)		7(3.5)		5(2.5)	
GA	332(28.9)	57(28.0)		54(27.4)		58(29.2)	
Allele							
G	1894(82.4)	335(82.5)	1.0(0.75-1.34)0.99	326(82.7)	1.02(0.76-1.36)0.96	328(82.8)	1.02(0.76-1.37)0.87
A	402(17.5)	71(17.4)		68(17.2)		68(17.1)	
RXR rs3132296							
Genotype							
AA	440(45.9)	85(42.9)	0.88(0.64-1.22)0.43	96(48.0)	1.08(0.79-1.49)0.6	82(41.8)	0.85(0.61-1.17)0.28
GG	78(8.2)	24(12.1)		20(10.0)		26(13.2)	
AG	439(45.8)	89(44.9)		84(42.0)		88(44.8)	
Allele							
A	1319(68.9)	259(65.4)	0.85(0.67-1.08)0.17	274(68.5)	0.98(0.77-1.25)0.87	252(64.2)	0.81(0.64-1.03)0.07
G	595(31.0)	137(34.5)		126(31.5)		140(35.7)	
RXR rs1045570							
Genotype							
GG	170(71.7)	153(72.1)	1.02(0.66-1.58)0.91	131(66.8)	0.79(0.52-1.22)0.27	136(71.2)	0.97(0.63-1.52)0.9
TT	5(2.1)	8(3.7)		7(3.5)		1(0.05)	
GT	62(26.1)	51(24.0)		58(29.5)		54(28.2)	
Allele							
G	402(84.8)	357(84.1)	0.95(0.65-1.39)0.87	320(81.6)	0.80(0.55-1.16)0.24	326(85.3)	1.04(0.7-1.55)0.82
T	72(15.1)	67(15.8)		72(18.3)		56(14.6)	
RXR rs4240705							
Genotype							
AA	312(38.7)	77(38.8)	1.01(0.72-1.40)0.96	82(41.8)	1.14(0.82-1.58)0.42	80(40.2)	1.06(0.77-1.48)0.69
GG	118(14.6)	34(17.1)		30(15.3)		32(16.0)	
GA	376(46.6)	87(43.9)		84(42.8)		87(43.7)	
Allele							
A	1000(62.0)	241(60.8)	0.95(0.75-1.20)0.66	248(63.5)	1.07(0.84-1.35)0.58	247(62.0)	1.00(0.79-1.26)0.99
G	612(37.9)	155(39.1)		142(36.4)		151(37.9)	
FXR rs1030454							
Genotype							
AA	150(70.4)	117(66.8)	0.85(0.56-1.33)0.44	138(70.7)	1.02(0.65-1.59)0.93	133(73.8)	1.19(0.74-1.90)0.44
GG	7(3.2)	7(0.4)		3(1.5)		3(1.6)	
AG	56(26.2)	51(29.1)		54(27.6)		44(24.4)	
Allele							
A	356(83.5)	285(81.4)	0.86(0.58-1.27)0.45	330(84.6)	1.08(0.73-1.60)0.68	310(86.1)	1.22(0.81-1.84)0.32
G	70(16.4)	65(18.5)		60(15.3)		50(13.8)	
FXR rs476498							
Genotype							
AA	194(20.1)	52(26.0)	1.39(0.96-2.01)0.06	49(25.0)	1.32(0.91-1.92)0.1	54(27.8)	**1.53(1.06-2.20)0.01**
GG	253(26.2)	50(25.0)		43(21.9)		41(21.1)	
AG	516(53.6)	98(49.0)		104(53.0)		99(51.0)	
Allele							
A	904(46.9)	202(50.5)	1.15(0.92-1.44)0.19	204(52.0)	1.23(0.98-1.52)0.06	207(53.3)	**1.29(1.03-1.62)0.02**
G	1022(53.0)	198(49.5)		188(47.9)		181(46.6)	
FXR rs7304328							
Genotype							
CC	638(67.2)	120(62.1)	0.80(0.57-1.12)0.1	127(66.1)	0.95(0.68-1.34)0.77	121(64.0)	0.87(0.62-1.22)0.39
TT	42(4.4)	15(7.7)		15(7.8)		13(6.8)	
CT	269(28.3)	58(30.0)		50(26.0)		55(29.1)	
Allele							
C	1545(81.4)	298(77.2)	0.77(0.59-1.02)0.05	302(79.0)	0.86(0.65-1.14)0.28	297(78.5)	0.84(0.6-1.1)0.2
T	353(18.5)	88(22.7)		80(20.9)		81(21.5)	
FXR rs7956050							
Genotype							
GG	364(91.0)	190(95.0)	1.88(0.87-4.14)0.09	166(92.2)	1.17(0.5-2.35)0.62	181(96.2)	**2.56(1.06-6.43)0.02**
AA	16(4.0)	4(2.0)		5(2.7)		5(3)	
GA	20(5.0)	6(3.0)		9(5.0)		2(1)	
Allele							
G	748(93.5)	386(96.5)	1.92(1.02-3.67)0.03	339(94.1)	1.12(0.65-1.96)0.79	364(96.8)	**2.11(1.07-4.22)0.01**
A	52(6.5)	14(3.5)		21(5.8)		12(3.1)	

5.3.2.3 Haplotype frequencies

Since these polymorphisms were chosen solely based on their ability to tag > 90% of the haplotypic variation in the region, analysis of the haplotype is of particular significance here. The haplotype frequencies of both *FXR* and *RXR* as calculated with PLINK and haploscore were significantly associated with the risk of oesophageal disease (both uncorrected and adjusted for age and sex using Haplostat), Table 17. A total of 30 tests were performed for all possible combinations in both pooled and separate populations (20 for individual population and 10 for pooled population groups). Even though, single SNP analysis failed to show a strong association with disease, indeed, haplotype reconstruction shows significant differences in the overall distribution of *FXR* haplotypes between the OAC, BO and RO population and the control population (p=0.000, 0.000 and 0.002 respectively) for the possible combinations.

FXR haplotype 314 for loci rs1030454 (A/G), rs476498 (A/G), rs7304328 (C/T) was significantly associated with RO and OAC with a reduced frequency in the disease group compared with controls. The *FXR* haplotype 114 for same loci (rs1030454 (A/G), rs476498 (A/G), rs7304328 (C/T) was significantly increased in BO compared with the controls. Similarly, the same haplotype (*FXR* haplotype 114) was at significantly increased frequency in the disease group (BO & OAC) compared with controls for pooled populations' analysis.

The *FXR* haplotypes 114 found to be at increased frequency in disease carry at least one major allele for the loci (rs1030454 (A/G), rs476498 (A/G), rs7304328 (C/T), whereas the *FXR* haplotypes 314 at a decreased frequency carry at least one minor allele for the same loci.

RXR haplotype 31 for loci rs1045570 (G/T) and rs4240705 (A/G) was significantly associated with OAC and RO. These haplotypes were at significantly reduced frequencies in the disease populations (OAC and RO) compared with the controls.

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5.3.2.4 Gene-gene interaction

A significant interaction was observed between *RXR* locus rs4240705 (A/G) and *FXR* loci rs476498 (A/G), rs7304328 (C/T) in the oesophageal disease patients compared with controls by cross-tabulation (SPSS 16 and PLINK).

When the stratified odds ratios (OR) are examined, the OR of *RXR* rs4240705 (A/G) allele 1 homozygote to allele 2 carriers is only significant in the presence of *FXR* rs476498 (A/G) allele 1 and allele 2 homozygotes for all cases (RO, BO and OAC as compared with controls). The allele 1 homozygote AA for *FXR* rs476498 (A/G) is significantly associated with OAC in the genotype analysis, (Table 18) whereas there was no difference in genotype distribution and allele frequencies for *RXR* rs4240705. These results show that even though *RXR* locus is not directly associated with disease, its interaction with its obligate partner *FXR* can result in disease susceptibility. Only one of the disease causing polymorphisms in the two genes is required to produce the association with disease. Similarly, *RXR* rs4240705 (A/G) shows significantly increased OR for disease in the presence of allele 1 homozygotes for *FXR* rs7304328 (C/T), Table 20. Both these loci have similar genotype and allele frequencies for cases and controls when considered individually. However, when the allele 1 homozygotes of both loci are observed together, a significant association with oesophageal disease is seen (BO and OAC). In addition, the effect seems to be additive, with alleles in both loci augmenting each other's effect whereas no effect is seen if the other locus is not polymorphic.

Table 17: Haplotype frequency of FXR and RXR

Cases	*FXR* haplotypes (rs103/476/73)	Total Haplotype frequency	Haploscore	p	*RXR* haplotypes (rs104/424)	Total Haplotype frequency	Haploscore	p
OAC	3 1 4	0.024	3.46	**0.000**	3 1	0.149	2.97	**0.002**
BO	1 1 4	0.0797	3.54	**0.000**				
RO	3 1 4	0.017	3.04	**0.002**	3 1	0.142	2.42	**0.015**
Pooled CON-RO/BO,OAC	1 1 4	0.087	2.602	**0.009**				

Table 18: *RXR* (rs4240705) A/G & *FXR* rs4764980 A/G Cross-tabulation

***FXR*rs476498 Genotypes**	***RXR*rs4240705**		**Controls**	**RO**	**BO**	**OAC**
11	11	Count	15	29	27	31
		%within group	10%	56.9%	56.3%	58.5%
	12+22	Count	135	22	21	22
		%within group	90%	43.1%	43.7%	53.7%
		Total	150	51	48	53
12	11	Count	140	14	16	13
		%within group	41.9%	14.6%	15.5%	13.3%
	12+22	Count	194	82	87	85
		%within group	58.1%	85.4%	84.5%	86.7%
		Total	334	96	103	98
22	11	Count	131	34	38	35
		%within group	55.3%	68%	90.5%	85.4%
	12+22	Count	106	16	4	6
		%within group	54.7%	34%	9.5%	14.6%
		Total	237	50	42	41

Table 19: Interaction between *RXR* (rs4240705) A/G & *FXR* rs4764980 A/G

***FXR*rs476498 Genotypes**	**OR (95%CI) for *RXR*rs4240705 (11/12+22)**	**p**
11	12.68 (5.01-29.38)	0.000000
12	0.25 (0.14-.47)	0.000002
22	4.72 (1.01-13)	0.0002

Table 20: *RXR* (rs4240705) A/G & *FXR* rs7304328 C/T Cross-tabulation

***FXR*rs7304328 Genotypes**	***RXR*rs4240705**		**Controls**	**RO**	**BO**	**OAC**
11	11	Count	168	70	76	74
		%within group	32%	58.3%	60.3%	61.2%
	12+22	Count	357	50	50	47
		%within group	68%	41.7%	39.7%	39.8%
		Total	525	120	126	121
12	11	Count	117	5	4	4
		%within group	50.9%	9.1%	8.3%	7.4%
	12+22	Count	113	50	44	49
		%within group	49.1%	90.9%	91.7%	92.6%
		Total	230	55	48	53
22	11	Count	19	0	0	0
		%within group	63.3%	0%	0%	0%
	12+22	Count	11	15	14	13
		%within group	36.7%	100%	100%	100%
		Total	30	15	14	13

Table 21: *RXR* (rs4240705) A/G & *FXR* rs7304328 C/T Cross-tabulationCross-tabulation

***FXR*rs7304328 Genotypes**	**OR (95%CI) for *RXR*rs4240705 (11/12+22)**	**p**
11	3.23 (2.12-4.92)	0.000000
12	0.08 (0.03-0.27)	0.00000
22	0.02 (0.00-0.26)	0.00007

5.4 Discussion

To our knowledge, this is the first study to examine the xenobiotic receptor polymorphisms in *PXR/NR1I2*, *FXR/NR1H4* and *RXR/NR2B1* genes in oesophageal disease. This study has highlighted the complexity of the genetic background underlying the inflammation related group of oesophageal diseases. Our results show that the orphan nuclear receptors FXR and RXR are associated with the aetiology of OAC and BO. Our study provides strong evidence of the association of inherited variation in the *FXR/NR1H4* gene on chromosome 9q and *RXR/NR2B1* with the pathogenesis of both BO and OAC based on the haplotype analysis (both uncorrected and adjusted for age and sex). Since these polymorphisms were chosen solely based on their ability to tag > 90% of the haplotypic variation in the region, analysis of the haplotype is of particular significance here. Even though, single SNP analysis failed to show a strong association with disease, indeed, haplotype reconstruction shows significant differences in the overall distribution of *FXR* haplotypes between the OAC, BO and RO population and the control population (p=0.000, 0.000 and 0.002 respectively) for the possible combinations. When the BO and OAC populations are pooled together as cases, the haplotype frequency against controls is highly significant again at p=0.009. The *RXR* haplotype frequency is significantly different in OAC (p=0.002). Along with the haplotype pattern, there is significant interaction between the *FXR* and *RXR*. Overall, these findings support accumulating evidence of a role for the xenobiotic response system in the development of oesophageal disease.

FXR rs7956050 and rs1030454 are located in the intronic region of *FXR* and *RXR* rs1045570 is located in the 3′UTR region of *RXR*. There is no functional data

published for these polymorphisms although intronic polymorphisms could affect splice sites or regulatory sequences. Also there is a large body of evidence that 3´UTR region of mRNA may contain regulatory elements that have important roles in post transcriptional regulation of gene expression like subcellular localisation [258], translation efficiency, transcript stability and mRNA transport out of nucleus [259]. It is therefore possible that this *RXR* polymorphism could have a functional effect. Alternatively, because the SNPs genotyped in this study are htSNPs they serve as markers for the actual haplotypes or SNPs that have a functional effect, as is shown by haplotype analysis and thus these SNPs may be linked to other functional polymorphisms anywhere in the linked region. Thus, in order to further investigate the cause of these associations, it will be necessary to undertake more extensive fine mapping and genotype analysis of potentially functional SNPs.

A significant interaction was observed between *RXR* loci rs3132296 A/G, rs4240705 A/G and *FXR* loci 476498 A/G, rs7304328 in the oesophageal disease patients compared with controls. The OR for allele 1 homozygotes for *RXR* rs4240705 (A/G) were significantly increased in the presence of allele 2 homozygotes for *FXR* rs476498 (A/G) and allele 1 homozygotes for *FXR* rs7304328 (C/T). Also, the OR for allele 1 homozygotes for *RXR* rs3132296 (A/G) were significantly increased in the presence of allele 1 homozygotes for *FXR* rs476498 (A/G). Given that RXR is an obligate binding partner of FXR, it is significant that we have uncovered evidence for lack of independence of these genes in patients with oesophageal disease. A possible functional interpretation of this observation is that in those patients who are homozygous for the disease associated allele of one gene, there is no requirement to have the disease associated allele of the other gene to have the functional effect which

promotes the disease state – the disease associated allele of either gene can abrogate the function of the PXR/FXR heterodimer. This represents the genetic evidence – however a definitive understanding and proof of the association would require the discovery of the causatively associated SNPs and an insight into the effects these have on the individual protein functions. In fact, *RXRα* is an obligate binding partner of *PXR, CAR* and *FXR* and therefore, has a critical role in xenobiotic and bile acid metabolism. While we did not observe any interaction of PXR with RXR, the PXR SNPs chosen here are not selected for haplotype tagging effects but rather as putative functional SNPs and thus the possibility of a similar interaction between these genes cannot be outruled.

Pro-survival genes including COX-2 and NFκB appear to be important in the Barrett's to adenocarcinoma pathway and one of the FINBAR studies has shown COX-2 8473 C allele to be significantly associated with OAC [190]. We have previously reported that NFκB, a transcription factor centrally involved in inflammation, tumourigenesis, and apoptosis, is sequentially activated from GORD through SIM to adenocarcinoma, and activated NFκB in cancer confers a worse prognosis [110, 192]. Intriguingly, a recent study has shown an interaction between the xenobiotic response system and NFκB regulated inflammatory mechanisms[260] . They found that *PXR* activation inhibited NFκB signalling *in vivo*, whilst higher constitutive expression of NFκB target genes and increased inflammatory infiltrate in the small bowel was found in *PXR* knockout mice [260]. Another study has demonstrated NFκB interaction with the *RXRα* DNA binding domain leading to a disruption of the *PXR/ RXRα* heterodimer [261]. Lord et al.[122] have previously shown that *RXR* expression is altered in BO and De Gottardi et al. [121] have linked increased expression of *FXR* in BO tissue.

Thus, a possible model for how the polymorphisms in *RXR* affect the aetiology of reflux linked oesophageal disease is that they result in, or are linked to, SNPs that result in decreased activity or expression of *PXR*, this leads to downregulation of CYP3A4 and other xenobiotic transporters lowering the ability of the gut lining to metabolise environmental toxins and other xenobiotics, possibly leading to a degradation of the mucosal wall, intracellular junctions and normal tissue physiology. At the same time, lower levels of *RXR* also appear to lead to loss of repression of NFκB mediated inflammation [260], leading to increased generalized inflammation particularly in the oesophagus.

In addition, further evidence of the link between the xenobiotic response system and the inflammatory response, is the finding that *FXR* appears to be involved in regulating some genes in the immune system, including IL-18 [262] and also is involved in the regulation of *PXR* expression. These nuclear receptors which have their highest expression in the intestine and liver, have also been shown to be expressed in cells of the immune system (in CD4 positive, CD8 positive, CD19 positive and CD14 positive cells) [263]. It is also clear that the innate immune system interferes with the xenobiotic response system, since it has been shown that LPS downregulates the expression of many important drug metabolizing genes such as the CYP450 3A family among others [264]. Thus nuclear receptors, as well as regulating the drug and xenobiotic response system, also seem to act as mediators of inflammation and the immunity. These studies support the conclusion made in the present study that there is complex interaction and crosstalk both within and between the orphan nuclear receptors, the xenobiotic response system and the inflammatory response.

The findings in the present study, along with the recent studies linking the xenobiotic response system and the inflammatory response, strongly suggest that *RXR* and *FXR* regulate an overlapping set of genes that encode enzymes involved in the elimination of xenobiotics, and display considerable cross-regulation of their target genes. The *RXR* haplotypes that were at a significantly different frequency in the OAC and BO patients compared with the controls may lead to altered *RXR* expression or activity which could have broad ramifications for the activities of all RXR's binding partners, with the potential for wide-spread effects on metabolism and the expression of immune genes involved in inflammation and antibacterial activity [262]. Thus, inherited variation in nuclear receptors could play a crucial role in regulating the balance between the two systems and there is the potential for a vicious cycle of increased toxic xenobiotics allied to an increased propensity to mount an inflammatory response leading to the inflammation-cancer sequence which underlies the pathophysiology of oesophageal disease.

Chapter 6
Discussion

6.1 Background

Oesophageal cancer remains one of the leading causes of cancer mortality in men, and is one of the top five causes of cancer-related mortality in black males [28]. Although the 5-year survival rate has improved markedly over the last few decades, it is still one of the poorest compared with other cancers. Fifty years ago, the commonest malignancy affecting the oesophagus was squamous cell carcinoma. Over the last three decades, however, the incidence of adenocarcinoma of the oesophagus has increased in many countries at a rate that exceeds that of any other malignancy [29-33]. The precise reasons for the rapid increase in the incidence of this tumour remain elusive. The striking geographical distribution and the racial disparity in the incidence of oesophageal cancer suggest the role of environment as well as genes in its causation. The rapidly rising incidence of OAC has led to an enhanced recognition of BO and GORD [39].

The epidemiology of oesophageal cancer has provided insights into genetic, nutritional, and other environmental factors that may modify the susceptibility of the oesophageal epithelium for neoplastic development. Several studies have indicated that oesophageal disease is indeed a complex multifactorial disease with probable underlying genetic component. To investigate the genetic variation associated with oesophageal disease, gene polymorphism association studies offer useful information and are an active and popular area of research. Previous studies from the FINBAR group have investigated an association between antioxidant enzyme genes including glutathione S-transferase P1 (GSTP1), manganese superoxide dismutase (MnSOD) and glutathione peroxidase 2 (GPX2) and BO or OAC [265]. Ferguson *et al.* have

reported an association of the inflammation regulatory gene COX-2 (8473 C allele) with OAC, using the FINBAR population based study [190]. Gough et al.. [188] demonstrated an association between functional SNPs in the interleukin-1 receptor antagonist gene and BO, and between the interleukin-10 gene and BO and OAC.

Thus the concept of inherited variation in oesophageal disease is an established one. However, the search for the identification of the actual genes responsible for carcinogenesis is still underway. There are many confounding factors such as inadequate study design, lack of knowledge regarding the polymorphic content and the behaviour of the genome that underlie the inability of studies to conclusively identify susceptibility genes.

In oesophageal disease, the role of bile acid induced inflammation and subsequent inflammation related injury to mucosa is well documented as the potential triggering factor for the origin of OAC. There is significant crosstalk between the inflammatory and immune pathways. Furthermore, obesity is a known independent risk factor for OAC. Therefore, the aim of the research presented in this study is to highlight and enhance the current genetic knowledge and understanding related to OAC and BO. We attempted to identify susceptibility loci for oesophageal disease in regions and genes regulating inflammation, immunity and bile acid (xenobiotic) metabolism.

6.2 Oesophageal disease and *IL18/IL18RAP*

To our knowledge, this is the first study to examine functional polymorphisms of the *IL18* gene and its receptor, *IL18RAP*, in patients with Barrett's oesophagus and oesophageal adenocarcinoma. We show that the polymorphism rs917997, which is linked to altered expression of *IL18RAP*, is strongly associated with both BO and OAC, at levels that approach genome wide significance and surpass this when cases

and controls are consolidated. SNPs in *IL18* are weakly associated with Barrett's oesophagus and OAC prior to correction for multiple testing. This points to the -607 promoter polymorphism as the most likely disease susceptibility locus in *IL18*, the C allele of which has previously been linked to upregulated *IL18* mRNA expression [178]. Here, the *IL18* –607C allele was significantly associated with the risk of Barrett's oesophagus and adenocarcinoma. The –607CC genotype was also significantly associated with the risk of Barrett's oesophagus and the –137G / –607C haplotype conferred an increased susceptibility to Barrett's oesophagus compared with the -607 allelotype alone and double homozygotes, CC at -607 and GG at -137, also showed increased risk of BO. Thus evidence at various levels supports this association. We found no evidence of genetic interaction of the *IL18* and *IL18RAP* loci, suggesting they act independently to confer disease susceptibility.

This study design is based on analysing candidate functional polymorphisms of the *IL18* signalling pathway in a step-wise biological progression of disease from reflux to cancer. Both the promoter polymorphisms -137 and -607 have been implicated in the regulation of gene expression of *IL18* [178].

IL-18, as a pro-inflammatory cytokine, is a good candidate for involvement in the pathogenesis of oesophageal disease, and this gene has already shown association with other autoimmune diseases such as type 1 diabetes [266], hence indicating that it can act as a potential pathogenic agent in such conditions. This study, however, provides the first evidence that there is a genetic association between *IL18/IL18RAP* and oesophageal disease, particularly for BO and OAC. Thus controls and patients with GORD represent similar genotypic make-up while BO and OAC together represent the other end of the disease spectrum. This is an important observation as

there is an urgent need to identify biomarkers of risk in BO. Most GORD patients with BO do not develop OAC, yet surveillance endoscopy with biopsy is recommended for the majority of patients diagnosed with the condition [193-195]. This approach has many drawbacks, including cost, limited evidence of effectiveness and patient concerns about risk of cancer. [195] The distinct pattern of expression of these polymorphisms in our study in Barrett's and cancer as compared with controls and reflux oesophagitis group suggests a critical molecular link that might explain the altered response to refluxate in the susceptible group with this polymorphism. Thus patients with reflux whose anti-tumour immunity in this underlying key biological pathway is altered via these polymorphisms are at risk of developing premalignant changes in their oesophageal mucosa.

6.3 Oesophageal disease and *TRB3*

The incidence of oesophageal adenocarcinoma has increased rapidly in the Western world and so has the prevalence of obesity. Epidemiological evidence strongly implicates obesity, raised BMI and metabolic syndrome as a risk factor in up to 40% of cases [49, 51, 123, 124]. TRB-3, a family of Tribbles proteins, is involved in impaired glucose metabolism and implicated in the metabolic syndrome. This is the first study to our knowledge to demonstrate the association of the *TRB3* gene with oesophageal disease especially oesophageal carcinoma. The Q84R variant, previously shown to be associated with modulating *in vivo* insulin resistance and related clinical outcomes, hereby shows a significant association with oesophageal carcinoma (OR 1.49, 95% CI, 1.02-2.14, p=0.02). Also, a high BMI 5 years prior to the interview date was associated with a 2.5 fold increased risk of oesophageal adenocarcinoma, which is similar to reports in other case-control studies [51, 52, 216-218].

Our findings from this study support the hypothesis linking insulin resistance, metabolic syndrome and oesophageal disease. Some studies have suggested that a high BMI is associated with an increased risk of Barrett's oesophagus [219-221], although Caygill *et al.* [221] suggested that obesity is only a risk factor for Barrett's oesophagus in young people. One possible mechanism for the association between BMI, BO and OAC has been suggested to be through the increased production of free insulin-like growth factor-1 (IGF-1) in obese subjects, which stimulates cell proliferation and inhibits apoptosis [222, 223, 224, 225]. TRB-3 has also been shown to interact with IGF-1/Akt pathway in neuronal cells, HepG2 cells and chondrocytes [226-228], although the exact details of this interaction are not clear.

These data provided the biological evidence that the *TRB3* R84 variant is a stronger inhibitor of insulin signalling as compared with the ''wild type'' (i.e., more common) Q84 variant and might play, therefore, a role in affecting insulin sensitivity [205], Akt inhibition and subsequent disease susceptibility.

6.4 Oesophageal disease and Nuclear Receptors

The nuclear receptors comprise a superfamily of ligand-activated transcription factors that, when activated, regulate an array of genes involved in the metabolism of xenobiotics. From this family, we selected the *PXR/NR1I2*, *FXR/NR1H4* and *RXRα/NR2B1* genes as these were previously studied in our unit to investigate association with Inflammatory Bowel Disease (IBD) [257] where we had shown significant associations of promoter polymorphisms in *PXR/NR1I2* with disease. Also, a number of interesting observations arising from the interaction and association analysis of *FXR/NR1H4* and *RXRα/NR2B1* htSNPs polymorphisms in IBD led us to

replicate these in the FINBAR cohort. As these polymorphisms were chosen solely based on their ability to tag the gene haplotype, our study provides strong evidence of the association of inherited variation in the *NR2B1* and *NR1H4* with the pathogenesis of both BO and OAC based on the haplotype analysis (both uncorrected and adjusted for age and sex). Therefore it is not possible to identify the possible causatively associated SNPs based on these findings but it is possible to identify the haplotypes on which they occur. Further work will be needed to identify the SNPs causing disease. Overall however, these findings support accumulating evidence of a role for the xenobiotic response system in the development of oesophageal disease.

This study also highlights the complexity of the genetic background underlying the oesophageal diseases, by showing significant interactions between the *NR2B1* and *NR1H4* genes. Of note, a significant interaction was observed between *RXR* loci rs4240705 A/G, *FXR* loci 476498 A/G and rs7304328 in the oesophageal disease patients compared with controls. The OR for allele 1 homozygotes for *RXR* rs4240705 (A/G) were significantly increased in the presence of allele 2 homozygotes for *FXR* rs476498 (A/G) and allele 1 homozygotes for *FXR* rs7304328 (C/T). Thus in subjects homozygous for one of the disease causative alleles, the functional effect of other disease associated gene is not required and either of the two genes can contribute to disease susceptibility. In fact, *RXR*α is a binding partner of *PXR, CAR* and *FXR* and therefore, has a critical role in xenobiotic metabolism because of its role as an obligate heterodimeric partner of nuclear receptors such as *PXR, CAR* and *FXR* etc. that regulate the expression of xenobiotic metabolising genes such as *CYP450, MDR1* and *MRP3*, underlying the cross talk between the nuclear receptors. However we did not observe any association with *PXR NR1I2* with disease which could be due to the selection of presumably functional SNPs rather than htSNPs.

Thus, a possible model for how the polymorphisms in *FXR* affect the aetiology of reflux linked oesophageal disease is that they result in, or are linked to, SNPs that result in decreased activity or expression of *RXR* and/or *PXR* [245] and this leads to downregulation of CYP3A4 and other xenobiotic transporters.

Further support for the role of xenobiotic metabolism comes from Lord et al. [267] who have previously shown that *RXR* expression is altered in BO and De Gottardi et al. [121] who have shown increased expression of *FXR* in BO tissue. At the same time, lower levels of *RXR* also appear to lead to loss of repression of NFκB mediated inflammation [260], leading to increased generalized inflammation particularly in the oesophagus. NFκB has previously been shown to be associated with oesophageal disease [192]. Thus, there is the potential for a vicious cycle of increased concentrations of toxic xenobiotics allied to an increased propensity to mount an inflammatory response. Thus, the findings in the present study, along with the recent studies linking the xenobiotic response system and the inflammatory response, indicate that inherited variation in nuclear receptors could play a crucial role in regulating the balance between the two systems.

6.5 Gene-gene interaction effects

Gene-gene interaction, or epistasis, is a possible reason for conflicting results between different genetic association studies [268]. Epistasis is the term used to describe the masking effect whereby an allele at one locus prevents the variant at another locus from manifesting its effect [269]. From a statistical point of view, epistasis refers to a deviation from additivity in the effect of alleles at different loci with respect to their contribution to a phenotype [270]. The presence of gene-gene interaction is a cause for concern in complex disease genetics since, if the effect of one locus is altered or

masked by the effects at another locus, power to detect the first locus is likely to be reduced and elucidation of the main effects at the two loci is hindered by their interaction. The effect of any gene-gene interactions between the different loci of the 6 genes was also investigated. Crosstabulation analysis methods were employed to investigate this hypothesis.

This proved to be particularly interesting with regard to *FXR/RXR* loci where a significant gene-gene interaction was observed in the four different population groups. Individually, the genotype and allele frequencies for most loci except *FXR* locus rs476498 were similar between cases and controls. However, in the presence of AA homozygotes for this locus, *RXR* locus rs4240705 A/G contributed significantly to disease susceptibility, strongly implicating interaction between the two genes.

6.6 Conclusion

This study has highlighted the complexity of the genetic background underlying the group of oesophageal diseases. The work presented here has shown the importance of the anti-tumour cytokine interleukin-18 and its receptor in the aetiology of oesophageal disease, in particular BO. Our study provides strong evidence of the association of inherited variation in the *IL18RAP* and *IL18* with the pathogenesis of both BO and OAC. The interaction analysis demonstrated a significant association between *FXR/RXR* and oesophageal disease (BO) seen in the four different phenotypes. Haplotype analysis of the *IL18, FXR* and *RXR* genes studied here revealed an association with BO and OAC. Overall, these findings support accumulating evidence of a role for the *IL18/IL18RAP* anti-tumour response system, genetic interaction between the xenobiotic response system and *TRB3* mediated insulin/Akt signalling in the development of oesophageal disease.

Thus the picture emerging from this research is that *IL18*, in conjunction with its receptor complex *IL18RAP*, may represent an important genetic pathway playing a role in the regulation of anti-tumour, anti-immunity and biodefense mechanisms in oesophageal disease. As there is a high degree of interaction between these inflammation-cancer sequence pathways at a cellular and protein level, disruption of this balance could therefore contribute to the profound chronic and relapsing inflammation that is seen in oesophageal disease (BO and OAC). Further research is required to conclusively elucidate the complex crosstalks between these regulatory pathways.

6.7 Future prospects

The results presented in this study highlight several future lines of investigation which may be followed. Genetic advances are progressing rapidly both in the knowledge obtained regarding the structure of the human genome, including the variation present and the interaction and linkage between these variants, and also in the technological advances available at present to study this variation and behaviour.

Continued recruitment of patients at St. James's hospital and at other centres across the country will be carried out to increase the sample size of the population under investigation, thus increasing the power of future studies. The recruitment of a large number of patients is a laborious task, particularly when the annual incidence of OAC is far less as compared with other cancers. However, it is crucial to the success of future genetic association studies as a large sample of patients is required to give sufficient numbers of the subphenotypic groups. In addition, higher sample numbers would better facilitate the interaction between genes to be examined. To date, the most successful identification of risk factors for various diseases has been where those risk factors have high odds ratios. As the task to identify the remaining disease risk factors which will most likely have small effect sizes on disease intensifies, patient and control sample sizes need to be increased substantially from those used in current studies to provide the power necessary to detect these small changes. This will only be achieved after extensive patient recruitment and possible collaborations between recruitment centres, given the disease incidence. The establishment of national biobank facilities, such as that proposed and currently being implemented in Ireland, is also an important and perhaps, necessary advancement for genetic association studies. Such projects will provide a pool of healthy control individuals sampled randomly throughout the country, from which to draw a control population for analysis.

The clinical impact of genetic findings is situated in a better understanding of the heterogeneity of oesophageal disease, in terms of location, age at onset and behaviour of the disease. Thus, along with the increased numbers of patient samples required, it is imperative to clearly define the subphenotypes of the disease. These include age at diagnosis, location of the disease, disease behaviour, extra-oesophageal manifestations, response to medical therapy and smoking habit. This would give greater power to detect specific genotype-phenotype correlations.

This study has provided some tentative positive associations between specific genetic variants. Among these positive disease associations are some genetic associations which have been identified for the first time in this study. *IL18* and *IL18RAP* have been shown to be significantly associated with disease. There is good evidence, based on the function of this gene as a proinflammatory cytokine, that these results are not false positive findings, and accumulating evidence from other diseases showing that the polymorphisms studied and found to be associated with disease in this study are actual functional variants which influence gene expression. Also, a strong association is seen between *TRB3* gene and OAC linking the previously suggested role of obesity, BMI and cancer. To confirm such results, these polymorphisms will need to be replicated in an independent population sample through collaborative works. It will also, in the future, be possible to re-assess this positive finding in a larger Irish patient sample after the continued recruitment of patients has been completed.

The future of genetic association studies to identify BO and OAC disease susceptibility genes looks promising. This study has identified a number of interesting candidates which may provide the direction for future functional and genetic studies.

International GWAS have been exceptionally successful in the detection of common genetic variants underlying a number of other complex human diseases, while less successful in others. Our research group are participating in an international GWAS being funded through the WTCCC2 consortium, and this will allow us to assess the contribution of these loci in an independent population of UK patients. Advances in the fields of genetics and oesophageal disease research will inevitably lead to the identification of, as yet unknown, oesophageal disease genes and reveal the molecular pathways at the heart of this disease.

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