Barrett's Esophagus:

New insights in the genetic patchwork of transdifferentiation and malignant transformation

Anouk Van de Winkel

Colophon

Financial support for this thesis was generously provided by:
Nederlandse Vereniging voor Gastroenterologie (NVGE)
Sectie Experimentele Gastroenterologie (SEG) van de NVGE
Afdeling Maag-, Darm- en Leverziekten, Erasmus MC Rotterdam
Greiner Bio-One B.V.

Layout by: Anouk Van de Winkel
Cover by: Paula Jones-Biesta & Anouk van de Winkel
Photography by: Deem Stam (www.deemfotografie.nl)
Printed by: Wöhrmann print service - Zutphen, The Netherlands

ISBN: 978-90-8570-785-1

Barrett's Esophagus: New insights in the genetic patchwork of transdifferentiation and malignant transformation

Barrett oesofagus: Nieuwe inzichten in de genetische lappendeken van transdifferentiatie en maligne transformatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 15 december 2011 om 9.30 uur

door

Anouk Van de Winkel
geboren te Roermond

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. E.J. Kuipers
	Prof.dr. M.P. Peppelenbosch
Overige leden:	Dr. K.K. Krishnadath
	Prof.dr. H.W. Tilanus
	Prof.dr. A.G. Uitterlinden
Copromotor:	Dr. L.J.W. van der Laan

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

A. van de Winkel

BARRETT'S ESOPHAGUS

Barrett esophagus is a premalignant condition of the esophagus, defined as displacement of the normal stratified squamous epithelium by specialized columnar lined epithelium.[1] It can be endoscopically detected as a salmon-colored segment and is confirmed in biopsy specimens after histological evidence of specialized intestinal metaplasia with presence of goblet cells. Barrett esophagus is the only commonly recognized risk factor for esophageal adenocarcinoma.[2-3] The incidence of Barrett's esophagus and esophageal adenocarcinoma have both increased rapidly since the 1970s and remain on the rise in the Western world.[4-5] Compared to the general population, Barrett's patients exhibit a 30- to 125-fold increased risk of esophageal adenocarcinoma.[5-7] Estimated annual risk ranges from a mere 0.2% to almost 3.5%.[8-10] Endoscopic surveillance is performed to detect early stages that lead to adenocarcinoma.[11-12] The current marker for incipient malignancy in the esophagus is dysplasia, defined as the neoplastic proliferation within epithelial glands without affecting the basal membrane. Neoplastic progression develops through a multistep sequence from intestinal metaplasia to low-grade dysplasia (LGD), high-grade dysplasia (HGD) until finally, adenocarcinoma.[7, 13-14] At present it is unclear which factors control the rate of neoplastic progression in Barrett's esophagus.[15]

Barrett's esophagus often arises as a consequence of mucosal injury from chronic gastroesophageal reflux, in which bile acids are an important toxic component.[7] A growing body of evidence suggests that the intrinsic adaptive response to the toxic bile acids from the gastroduodenal contents is unable to prevent injury to the esophageal lining, thus suggesting a role for bile-induced signaling in the progression of Barret's esophagus. Therein, nuclear receptors as described in bile-induced signaling may be of vital importance in Barrett's esophagus.[16] However, the exact role of bile acids in the pathogenesis of Barrett esophagus remains controversial. Moreover, while the importance of acid and bile exposure in the development of Barrett's esophagus is well established, only a small percentage of Barrett's patients will ultimately develop an esophageal adenocarcinoma.

DIAGNOSIS AND TREATMENT

Given that regurgitating bile and acid reflux from the stomach inflicting severe caustic chemical injury to the esophageal mucosa is thought to be a major contributor to the development of Barrett's esophagus, most treatment strategies focus on acid-blocking medicines. Unfortunately, this has had unfulfilling success rates as no convincing evidence has been reported of effects of longstanding acid-suppressive treatment on the incidence of esophageal adenocarcinoma in Barrett's esophagus.[17]

Diagnosis of Barrett esophagus still relies heavily on the findings from endoscopic biopsy samples, and a great number of random esophageal biopsies are needed to detect specialized intestinal metaplasia. Current biomarkers are not applied in clinical practice due to lack of reliability, which are vital to improve the diagnosis of dysplasia in Barrett's patients.[18-20] Additionally, agreement between pathologists is poor due to intraobserver and interobserver variation.[21-22] Thus, BE patients are left with surveillance programs until dysplasia is identified, at which time treatment strategies are often scant and insufficient.

It is not known whether Barrett esophagus is a hereditary condition. BE is believed to be a complex disease and indeed no single causative gene has been identified. The combination of polymorphisms and genetic variations could potentially create a predictive screening model for identifying patients at risk for Barrett's esophagus and esophageal adenocarcinoma from the general population, but sensitive genetic predictors are yet to be discovered. A considerable problem herein is that half of patients with esophageal adenocarcinoma do not experience any symptoms until diagnosis.

There is a lot of attention for developments in the field of diagnosis and treatment of BE and esophageal adenocarcinoma, which will probably continue given the persistent rise in the incidence of these malignancies. Key questions for future research should include; which diagnostic biomarkers can be employed in clinical practice, can we discover safe chemopreventive agents, is capable of screening the general population for BE, and how can we personalize treatment recommendations and chemopreventive therapies for patients that will benefit most. Moreover, there is a strong incentive to undertake more basic science research into the pathogenesis of BE, as this could help the development and improvement of chemoprevention strategies.

PATHOGENESIS OF BE

The field of Barrett esophagus is evolving, with advances in enhancing treatment and diagnosis and understanding of pathogenesis underlying this premalignant condition. Over the past decade, several hypotheses elucidating the progression from squamous epithelium to intestinal metaplasia have been proposed. The crucial role that developmentally relevant transcription factors have in the pathogenesis of Barrett esophagus is emerging, and accordingly current research focuses on the cellular and molecular mechanisms that are involved.

Searching out for causative genes in the pathogenesis of Barrett's esophagus, the question emerges why the esophagus of some patients responds to chronic reflux by transdifferentiation to fully differentiated BE cells instead of simply regeneration of the squamous tissue lining the esophagus. This has drawn attention upon morphogenes that are capable of steering the phenotypic fate of cells. Indeed it has

been advised current investigations should focus on such genes that are ordinarily turned off during embryogenesis when the columnar lined esophagus changes to squamous lined as normal in adults.[23] Reactivation either in a programmed fashion or in response to exposure to a toxic environment may be associated with the manifestation of BE. Such genes with morphogenetic abilities thought to have a key role in the pathogenesis of Barrett's esophagus include those belonging to the families of Caudal-related homeobox (CDX), Bone morphogenetic protein (BMP), and Homeobox (HOX) genes. Abnormal genetic expression of CDX2, resulting from epigenetic changes, has been shown to be associated with the development of Barrett esophagus.[24] Animal models have also shown that exposure to duodenal contents induces the expression of CDX2.[25] The importance and functional role of bone morphogenetic protein 4 in the pathogenesis of Barrett esophagus has also been studied. Development of Barrett's metaplasia is proposed to result from bile and acid-induced successive events including signaling through stromal factors and expression of intestine-specific genes.[26-27] In contrast, even though it is very conceivable that HOX genes, being master regulators of processes involved in cell and tissue differentiation, are involved in Barrett's pathogenesis, knowledge herein from basic research is lacking. Presumably, this is in partly due to the fact that are not well studied in humans adult tissue overall, and from that they are practically challenging genes to study.

AIM AND OUTLINE OF THIS THESIS

As outlined above, premalignant Barrett's esophagus is the only commonly recognized risk factor for esophageal adenocarcinoma. It is a highly aggressive and morbid cancer, and its incidence is still on the upward trend despite many efforts to improve screening and surveillance of BE, and advance chemoprevention in esophageal adenocarcinoma. The importance of acid and bile exposure in the development of BE is well established, but strikingly, its precise mechanism of action remains disputed and processes underlying BE development as well as causative genes involved are poorly understood. Hence, the studies that give shape to this thesis aimed to gain new insights into the etiology of Barrett's esophagus, a genetically heterogeneous and complex disease, defined by an largely understood process of continuous transdifferentiation until malignant transformation occurs. A detailed treatise on the development of this work during the thesis research is given in the general discussion and conclusion (**Chapter 9**). In short this thesis starts with an overview and update of current status of clinical as well as preclinical research in Barrett's esophagus and associated esophageal adenocarcinoma is reported in a meeting report of DDW 2011, enclosed in **Chapter 2.** To begin to understand the pathways involved in development of BE, we firstly focused on the role of bile acids and nuclear receptors. **Chapter 3** explores the role of the nuclear Pregnane X Receptor (PXR) in BE and associated esophageal adenocarcinoma. **Chapter 4,** reports on the clinical value of PXR combined with bile acid Farnesoid X Receptor

(FXR) as a diagnostic tool in improving the accuracy of grading dysplasia. **Chapter 5** reports striking results from a genetic polymorphisms study of the Vitamin D Receptor in Barrett's esophagus and esophageal adenocarcinoma including evidence for functional implications. In **Chapter 6,** we touch upon the role of *HOXA* genes in anteroposterior patterning. **Chapter 7** presents data from a genome-wide association study on susceptibility to Barrett's esophagus executed in Dutch and UK populations. **Chapter 8** describes the effect of bile acid-dependent expression of FXR on the immune response in BE. Finally, in **Chapter 9** findings of this thesis that have lead to new insights are discussed and incorporated in a proposed model for the molecular mechanism leading to the manifestation of Barrett's esophagus.

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CHAPTER 2

DIGESTIVE DISEASE WEEK 2011: HIGHLIGHTS OF CLINICAL AND PRECLINICAL RESEARCH ON BARRETT'S ESOPHAGUS AND ASSOCIATED ESOPHAGEAL ADENOCARCINOMA

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Conditionally accepted in: Diseases of the Esophagus

ABSTRACT

Clinicians and basic researchers worldwide convened at the annual Digestive Disease Week (DDW) where the latest research in the field of gastroenterology and hepatology is presented. In this report, we summarize highlights of the convention on the field of Barrett's esophagus (BE) and associated esophageal adenocarcinoma (EAC). We reflect on new clinical and preclinical developments in etiology, diagnosis, surveillance, and prevention and therapy of BE and EAC in respect to current knowledge. We also discuss the relevance and impact of these findings on the future of BE and EAC research.

INTRODUCTION

Barrett's esophagus (BE) is defined as an acquired condition in which the stratified squamous epithelium of the lower esophagus is replaced by specialized intestinal epithelium. Patients with BE have a 30- to 125-fold increased risk of esophageal adenocarcinoma (EAC) relative to the general population.[1, 2] EAC portrays a poor prognosis with an approximate 85% 5-year mortality. The incidence of BE and EAC are both rapidly increasing.[3] These epidemiological developments in combination with the poor prognosis of EAC and the evolution of new detection methods[4] and rapid improvements in endoscopic treatment methods for BE[5, 6] together initiate research into screening for BE and early neoplastic lesions. Such initiatives are for now hampered by the low incidence of the disease and the significant costs of screening methods, impairing the cost-efficacy of population-wide. Currently, endoscopic surveillance is performed in patients with known BE to detect early stages of dysplasia. As annual progression rates in BE to HGD or EAC are estimated at a mere 0.4 to 1.0%,[7, 8] there is a need for markers that help to identify BE patients at risk for neoplastic progression. Without information on predisposing factors, patients can only wait until dysplasia is identified, at which time endoscopic treatment strategies are often insufficient and surgical interventions are highly morbid.

The complex and multifactorial character of BE etiology makes prevention strategies for EAC challenging. If we better understand the pathogenesis of BE and reflux esophagitis and study its underlying molecular mechanisms and signaling pathways, ensuing research can then focus on defining sensitive biomarkers for diagnosis of BE and may offer novel therapeutic strategies for the prevention of EAC. Recent progress in research was presented at the DDW.

A total of 15,566 clinicians, researchers, and scientists from around the world attended and presented their findings at the DDW 2011, held this May in Chicago, IL. The latest data on BE and EAC were presented in 26 sessions, focusing on BE, GI endoscopy, and chemoprevention and therapy. These include 167 abstracts that presented on Barrett’s esophagus and esophageal cancer specifically. Here, we review the major findings and highlights of these presentations, and report on excellent state-of-the-art summaries on markers of progression, risk stratification, and management of BE. We address the clinical and preclinical aspects of etiology, diagnosis, prediction, biology, surveillance, biomarkers, and treatment strategies. Abstracts highlighted in this report are indicated in Table 1.

Table 1. Abstracts highlighted in this report concerning etiology, diagnostics, surveillance, treatment strategies, and prevention of BE and EAC

Highlights on the topic of	No. of abstracts used	References
Etiology	Clinical: 3	[15], [19], [22]
	Preclinical: 5	[23], [25-26], [27], [28]
Diagnostics	Clinical: 2	[30], [31]
	Preclinical: 2	[36], [37]
Surveillance	Clinical: 2	[38], [39]
	Preclinical: 6	[40], [41], [42-43], [43], [46]
Treatment strategies	Clinical: 6	[47], [48], [49], [50], [51], [52]
	Preclinical: 6	[53], [57], [58], [60], [61], [62]
Prevention	Clinical: 2	[66], [67]

RESEARCH HIGHLIGHTS ON THE ETIOLOGY OF BE AND EAC

There is a striking geographic variation in the prevalence rates of BE. BE is much more common in the West when compared with Africa and most parts of Asia.[9] However, in some Asian countries like Japan and Singapore, lifestyle changes have also resulted in an increase in the incidence of BE and EAC.[10, 11] The demographics of BE are well established, being found in older Caucasian men with gastroesophageal reflux disease (GERD) symptoms for over 10 years.[8] BE develops as a combination of genetics and environmental factors. Specific genes involved and identification of particular environmental factors are still being elucidated. The latest observations in obesity and dietary factors will be discussed in more detail here, followed by a summary of current efforts into identifying causative genes in BE.

CLINICAL:

DIETARY FAT INTAKE, METABOLIC SYNDROME, AND ADIPOKINES

The simultaneous rise in incidence of EAC and in obesity in the Western world during the last decades indicates that obesity is an important etiological factor for the development of EAC.[12] Overweight (BMI >25 kg/m^2) and obese individuals (BMI >30kg/m^2) are twice as likely to develop EAC as individuals of normal weight and it has been estimated that around 40% of EAC will eventually be caused by obesity if its incidence keeps increasing.[13] Thus, adiposity is important, prompting delineation of its interaction with esophageal disease.

Previous studies suggested that in addition, the intake of dietary fat may be involved in the development of EAC.[14] This hypothesis is supported by a prospective cohort

study, where 23,500 healthy men and women were asked to keep food diaries over 7 days.[15] The diaries were coded by nutritionists, and participants were followed to identify those with a new diagnosis of BE or EAC. Eighty participants were newly diagnosed with BE and a further 58 developed EAC. The risk of EAC was positively associated with a higher fat intake and saturated fat intake, but an association between BE and dietary fat intake was not detected. Therefore controlling dietary fat intake may be useful once BE has already been established to prevent progression to cancer. Obviously delineating the molecular mechanisms involved will prove exceedingly useful in efforts to halt the increase in EAC incidence.

There is increasing evidence that the body-fat distribution may be even more relevant than a high BMI alone in the risk of developing BE and EAC. Studies from recent years have suggested that most of this risk might be mediated by visceral abdominal tissue (VAT).[16, 17] Abdominal obesity may lead to an increased gastric reflux due to high abdominal pressure and thus promote the development of BE and further EAC. Apart from the mechanical aspect, the involvement of adipokines has been regarded as an important factor in cancer development and progression in recent years.

Adipose tissue is nowadays regarded as an active endocrine organ. Visceral adipose tissue is known to be more metabolically active than subcutaneous adipose tissue[18] and seems to be an important link to the metabolic syndrome: obesity, glucose intolerance, hypertension and dyslipidemia. However, with the exception of obesity, the components of the metabolic syndrome and their relationship with BE have so far not been studied extensively. These associations were recently determined in a population based case-control study. One-hundred-and-three cases with BE, 103 controls with GERD, and 103 controls without GERD were matched for age, sex and duration of follow up. Information on BMI, hypertension, diabetes and hyperlipidemia were collected from medical records and metabolic syndrome turned out to be associated with a risk of BE nearly twice as high as in controls.[19]

Dysfunctional adipose tissue in obese individuals may lead to an increased production of inflammatory cytokines and alterations in adipokines. Simultaneously with the rise of obesity, the amount of macrophages infiltrating the adipose tissue increases, resulting in an increased production of adipokines and inflammatory cytokines. The altered production of adipokines and the chronic inflammation of the visceral adipose tissue may influence the microenvironment of a tumor. In recent years studies have found that adipokines may influence proliferation and apoptosis in BE mucosa and EAC.[20, 21] To investigate whether a differential production of omental adipokines provides further clues with regard to possible mechanisms involved in the development of esophageal adenocarcinoma, our research group collected samples from omental adipose tissue of patients with EAC and controls. In addition to a high expression of visfatin and resistin in the adipose tissue of cancer

patients, we also found a higher IL-10 expression in omental adipose tissue in cases than in that of controls.[22] While we had included only small numbers of patients so far, these preliminary results might support the hypothesis that a high secretion of the IL-10 in patients with EAC may create an immunosuppressive state through a shift from cell-mediated (TH1) immune response to humoral immune response (TH2) in favor of tumor development. IL-10 inhibits cytotoxic T-cells which may favor tumor development and tumor escape from the immune system. Whether serum IL-10 is high in patients with EAC and whether this may be a starting point for future therapy needs to be evaluated in further studies (see Figure 1).

PRECLINICAL:

BE can develop as a complication of reflux esophagitis, which is a result of caustic chemical injury of esophageal mucosa by bile, acid and pepsin regurgitating from the stomach. The importance of acid and bile exposure in the development of BE is well established, and for years therapeutic strategies have been directed at prevention and reversal of caustic injury. As attempting to prevent injury to the esophageal lining has had unfulfilling success rates, researchers are unavoidably confronted with the question: "What if acid is not it?" (Quoting Dr. Souza). This question leads to believe that future research and therapeutic strategies should focus on the process of BE development. The discovery and further investigation of causative genes will be the first step in this.

In exposing these causative genes another question has puzzled most BE researchers: "Why does, in some patients, damage to esophageal cells caused by GERD heal through transdifferentiation to fully differentiated BE cells rather than by regeneration or a temporary switch?" In search for genes predisposing Barrett's metaplasia, attention draws upon morphogenes that are capable of steering the phenotypic fate of cells. They are commonly expressed in the development of columnar epithelium during embryogenesis. Many current investigations focus on such genes that are ordinarily turned off during embryogenesis when the columnar lined esophagus changes to squamous lined as normal in adults. Reactivation may be associated with the manifestation of BE. This interest in genes belonging to the families of CDX, BMP, and SOX genes was also apparent at DDW 2011 (see Figure 1).

CDX2

CDX2, a transcription factor that regulates the development of an intestinal cellular phenotype, is expressed by Barrett's epithelial cells. In human esophageal squamous cell lines derived from GERD patients with BE, acid and bile salts have been shown to cause nuclear translocation of both the p50 and p65 subunits of NF-κB, but only p50 binds the CDX2 promoter to induce CDX2 expression. In esophageal squamous

cell lines from GERD patients without BE, in contrast, acid and bile salts cause no increase in CDX2 expression. This suggests that differences in reflux-induced activation of NF-κB pathway proteins may underlie differences in CDX2 expression between esophageal squamous cells from GERD patients with and without BE. To explore this hypothesis further, Huo *et al.* studied the effects of acid and bile salts on NF-κB pathway proteins in telomerase-immortalized esophageal squamous cell lines derived from patients who had GERD with and without BE.[23] Cells were exposed to acidic media, neutral bile salt media, or acidic bile salt media. Esophageal squamous cell lines derived from GERD patients with and without BE exhibit substantial differences in NF-κB activation. Compared to squamous cells from BE patients, squamous cells from GERD patients exposed to acid and bile salts exhibit less phosphorylation of IKKα/β and IκB, and less nuclear translocation of p50. Unlike the squamous cells from Barrett's patients, furthermore, the GERD squamous cells show no nuclear translocation of p65, and p50 does not bind their CDX2 promoter. These original findings suggest that phenotypic differences among patients in how the NF-κB pathway in their esophageal squamous cells responds to GERD injury might determine whether they develop Barrett's metaplasia (see Figure 1).

BMP4

Previous studies showed that Bone Morphogenetic Protein 4 (BMP4) is reactivated in esophagitis and BE. The BMP pathway is proposed to be one of the most important signaling routes involved in the development of Barrett's metaplasia.[24] At DDW, data were presented on the effect of Noggin treatment, an endogenous BMP inhibitor, in a surgical rat esophagitis model.[25] A trend was found towards decreased BMP pathway activity in the Noggin treated rats as compared to the nontreated groups, showing that 4-day oral treatment with Noggin tends to inhibit the BMP pathway in a surgical rat model for reflux esophagitis, though this observation did not reach statistical significance. Next to the BMP4 pathway, CDX2 and CDX1 transcription factors seem critical for acquisition of specialized intestinal epithelium characteristic for Barrett esophagus. Another study reported that the specific intestinal columnar phenotype of BE is induced through a direct cooperative interaction of CDX-2 and the BMP4 downstream targets PSMAD 1,5,8.[26] Roman *et al.* reported their results on the involvement of the BMP4 pathway and CDX2 expression in nonspecialized columnar epithelium,[27] which has been proposed as an intermediate stage in the transdifferentiation process from normal squamous into specialized intestinal epithelium present in Barrett esophagus (see Figure 1). Hereto, biopsy samples were taken from the remnant esophagus of patients that underwent esophagectomy at different time points after surgery. Expression and early activation of BMP4 was identified in squamous epithelium and nonspecialized columnar epithelium. Scattered nuclear expression of CDX2 was observed in nonspecialized columnar epithelium, but not CDX1 expression before the

appearance of the intestinal phenotype. However, they fell short in establishing a model for specialized columnar metaplasia as they had only found two patients with metaplasia with few goblet cells so far.

SOX9

SOX9, a target of the BMP/SMAD pathway, is a transcription factor that regulates the development of columnar cell morphological features. In human esophageal squamous cells, the forced expression of SOX9 induces the expression of cytokeratins 8 and 18 and the development of a columnar phenotype. This suggests that, like BMP4, expression of SOX9 by esophageal squamous cells may precede the development of Barrett's metaplasia. Wang *et al.* presented their findings on SOX9 and two of its target genes namely columnar cytokeratins 8 and 18 in before mentioned squamous cell lines derived from patients who had GERD with BE, and without BE.[28] All cell lines expressed the squamous cytokeratins 4, 5, 13, and 14. In contrast, only the squamous cells from the patients with BE also expressed columnar cytokeratins 8 and 18 and the same was found for SOX9 expression. In addition, knockdown of SOX9 by siRNA decreased expression of cytokeratin 8 substantially in the NES-B3T and NESB10T cells. These findings support the hypothesis that phenotypic differences in SOX9 expression can identify which patients are prone to develop Barrett's metaplasia in response to GERD injury. This prompts SOX9 a promising early marker of BE.

RESEARCH HIGHLIGHTS ON DIAGNOSTICS

For the diagnosis of BE columnar epithelium lines must be present in the distal esophagus and second, the histological examination of biopsy specimens from the columnar epithelium must reveal intestinal metaplasia. However, these diagnostic criteria are not very robust. There is a high interobserver variability in defining the length of BE and in addition, intestinal metaplasia may not always be evident on endoscopy.

CLINICAL:

PRAGUE C & M CRITERIA

The Prague C & M Criteria (2007) have been recommended for assessing the presence and extent of Barrett's esophagus. The criteria are based upon assessment of the circumferential extent (C) and maximum extent (M) of intestinal metaplasia

above the GEJ. A high interobserver agreement for BE length > 1cm was found in the primary validation study[29] and was now confirmed by Herrero *et al.*[30] who detected a high interobserver agreement for BE length when comparing a group of experts with non-experts during real-time endoscopy in a randomized cross-over trial. There were no differences in the levels of agreement between expert and non-expert endoscopists. The investigators also observed a reasonable interobserver agreement for hiatal hernia length. This study thus corroborates the validity of the Prague C & M criteria for assessing BE and might help determine whether these criteria will be used broadly in the future or not.

OVER-DIAGNOSIS OF BE

The diagnosis of BE may sometimes be challenging when a large hiatal hernia is present. Furthermore, it may be occasionally difficult to distinguish goblet cells from gastric cardiac cells. These factors may lead to an over-diagnosis of BE and unnecessary enrollment of patients in a surveillance program. A group of researchers from Minnesota showed in their cohort study that that BE was over-diagnosed in 33% of patients previously diagnosed with BE.[31] Of 112 patients, 37 of them had their diagnosis reversed when endoscopy was repeated and results reviewed. The diagnosis was changed because either there was no columnar-lined epithelium proximal to the gastric folds or because no goblet cells were found on biopsy. Future studies should focus on standardizing the diagnosis of BE to avoid over-diagnosis.

PRECLINICAL:

Many attempts have been made to establish a useful rodent model of BE and EAC, including several surgical and one bile acid feeding model.[32, 33, 34, 35] In the past decade, a model of esophagojejunostomy to induce BE and EAC in rats has been used and characterized. Notably, over 90 presentations at the DDW presented on animal models in GI and liver disease, where only few reported to have established a BE or BE-like animal model.

ANIMAL MODELS

At the DDW, a group of researchers presented on their model of bitransgenic mice that were engineered to mimic oncogenic Hedgehog signaling in stomach, using an activated form of the transcription factor GLI2.[36] These mice carried a Cre-inducible GLI2 active transgene under the control of a ubiquitous promoter and a K5-Cre transgene which drives recombination in forestomach. These K5- Cre;CLEG2 mice, in which GLI2 expression was activated in forestomach squamous epithelium, developed a dysplastic, Barrett's-like epithelium near the squamocolumnar junction.

While most of the abnormal cells expressed glandular markers keratins K8 and K19, a subset of cells co-expressed squamous markers keratin K5 or K14, supporting the idea that the dysplastic Barrett's-like lesions are derived from Hedgehog-responsive basal cells in the forestomach. Unfortunately, this study does not show the expression of markers specific for Barrett's metaplasia or goblet cells. In investigating the effects of melatonin on BE, data was presented from eighty surgically prepared non-pinealectomized and pinealectomized rats with esophagogastroduodenal anastomosis, that resulted in chronic esophagitis resembling BE in humans.[37] In these rats, excessive release of TNF-α and IL-1β was observed. Furthermore, esophageal blood flow was impaired due to overexpression of COX-2 and iNOS. These alterations were more pronounced in pinealectomized animals. Histology revealed extensive esophageal ulcerations with development of columnar epithelium, formation of mucus glands in squamous epithelium, intestinal metaplasia distant to anastomosis consisting of goblet cells, and infiltration of inflammatory cells. They further show that treatment with exogenous and endogenous melatonin exerts beneficial effects in this model by activating Mel2 receptors, suppressing TNF-α and IL-1β release, and attenuating the proinflammatory markers COX-2 and iNOS in esophageal mucosa. Moreover, treatment with pantoprazole also had a beneficial effect by reducing neutrophil infiltration of the esophageal mucosa, while causing significant rise in the esophageal blood flow and expression of Mel2 receptors. This indicates that acid suppressive drugs such as pantoprazole could be useful in the treatment of BE.

RESEARCH HIGHLIGHTS ON BE SURVEILLANCE

BE is the most established risk factor for development of EAC, and about 10-20% of individuals with BE will develop dysplasia at some time. However, annual progression to HGD or EAC in BE patients is estimated around 1%. The current marker for incipient malignancy in the esophagus is grade of dysplasia. Periodic endoscopic biopsy surveillance in patients with BE is necessary to detect dysplastic changes in an early stage. The presence and grade of dysplasia in biopsies obtained during endoscopy is currently the best marker for malignancy. Unfortunately, endoscopic biopsy surveillance is subject to misclassification caused by sampling bias and limited interobserver agreement amongst pathologists. Therefore, there is a great need for better biomarkers for progression.

CLINICAL:

SAMPLING BIAS AND MISCLASSIFICATION

A Dutch population-based study described the prevalence of sampling bias and misclassification in patients diagnosed with HGD in BE between 1999 and 2008.[38] Sampling bias or misclassification was defined as more than one histological evaluation following HGD diagnosis which was scored as less severe than HGD, including both biopsies and resection specimens. Of 515 patients included with HGD in BE, in more than half sampling bias or misclassification was involved during subsequent follow-up evaluations. The risk of re-detecting HGD during endoscopic follow-up was increased when performed in a university hospital and it decreased with increasing numbers of histological evaluations with a diagnosis less severe than HGD. It is important to account for these factors when determining the follow-up strategy of patients in which HGD is detected.

AGREEMENT AMONG EXPERT PATHOLOGISTS ON DYSPLASTIC CHANGES

Among experienced pathologists, interobserver agreement for the diagnosis of LGD is below 50%. A consensus diagnosis of LGD among expert GI pathologists has furthermore been associated with an increased risk of progression to HGD and EAC. Surprisingly little progress had been made on this topic. A multicenter cohort study of 210 patients with BE with LGD also presented a very low reliability of diagnosing LGD even among expert GI pathologists.[39] Also, a consensus diagnosis among the expert pathologists was not associated with increased rates of progression. These results highlight the dilemma in the diagnosis and management of LGD.

PRECLINICAL:

BIOMARKERS FOR PROGRESSION

In light of these endoscopic limitations and disadvantages and conflicting pathologic interpretation, the search for novel, accurate prognostic biomarkers is indispensable to inform clinical decisions. A considerable number of biomarkers have previously been suggested, including tumor markers and markers identifying genetic abnormalities (aneuploidy), chromosomal loss, DNA hypermethylation, and aberrant proliferation and cell cycling. To date, none are ready for clinical application. At the DDW 2011, Bansal *et al.* presented their findings on the natural variation in the expression of four known molecular markers within Barrett's segments.[40] Of the panel of proliferation markers ki67 and MCM2, and cell cycle markers cyclin D1 and A, cyclin A had the lowest variability within the BE segment, suggesting it could be a good biomarker for intervention trials with the need for only

a few representative biopsies. A major downside for most BE patients is the complete absence of surface expression of cyclin A and other biomarkers in over 90% of the patients.

In addition, several novel biomarkers were presented. A study of aberrant methylation in neoplastic progression in BE found that SFRP4 and vimentin may represent markers which distinguish non-neoplastic BE from neoplastic stages.[41] Also, Egr-1 was suggested as a novel biomarker of EAC.[42] P504S, CD133, and Twist, were evaluated in another study including 25 cases each of BE, LGD, and EAC, along with 25 cases of esophagectomy resections for Barrett's EAC.[43] P504S did not express in any case of BE. However, its expression was significant in LGD, EAC and resections. CD133 also did not express in any of the BE or LGD. Its comparative expression was upregulated in cases of EAC and resections. Twist expression was weak in BE and LGD but over-expressed in cases of EAC and resections. This cross sectional study has shown increased expression of P504S, CD133 and Twist in the metaplasia-dysplasia-adenocarcinoma sequence and has suggested their possible role as potential biomarkers of Barrett's progression.

Other recent studies have presented models that combine several biomarkers. A model of eight methylation biomarkers predicted 50% of progressors to HGD and EAC that would not have been diagnosed earlier without using these biomarkers,[44] and our group show a model of FXR and PXR to have potential value as a diagnostic tool.[45] To accomplish sufficient accuracy for clinical use, these types of studies need refinement to enhance sensitivity and specificity. Moreover, current studies are often transversal and focus on diagnosis and grading, yet greater clinical merit lies in large prospective follow up studies to investigate risk prediction in early stages of EAC.

RISK PREDICTION: LENGTH OF THE BE SEGMENT

There is substantial momentum driving the notion that the length of the BE segment correlates well the risk for disease progression. Individuals with long segments of BE (≥3 cm) seem to be at higher risk of developing dysplasia and EAC than those with shorter segments (<3 cm). However, most of the studies performed so far were of retrospective design. Gaddam *et al.*[46] presented their results of a multicenter cohort study of 3599 non-dysplastic BE patients to quantify the risk of HGD and EAC in relation to the length of the BE segment. Mean BE length was 3.8 cm. In a multivariate logistic regression model they corrected for age, race, sex and smoking and found a higher risk of progression to HGD and EAC in individuals with longer BE segments than in those with a short BE. Per 1 cm increase in BE length they calculated a 21% increase in risk of HGD or EAC. The most straight-forward explanation is that further transformation of BE in to more malignant phenotypes is a random process and that a longer segment entails a greater risk for such an event.

Nevertheless, life style factors greatly influence the probability of such an event, especially those related to adiposity. In spite of the large cohort, the retrospective design certainly is a disadvantage of this study.

RESEARCH HIGHLIGHTS ON TREATMENT STRATEGIES

Dysplastic and early cancerous lesions can nowadays be resected using endoscopic methods. The main options for treatment of HGD are esophagectomy, endoscopic mucosal resection (EMR), and endoscopic ablation (radiofrequency ablation (RFA), photodynamic therapy, or cryotherapy). The American Gastroenterological Association recommends in its guidelines from 2011, that patients with HGD undergo endoscopic eradication therapy with RFA, photodynamic therapy, or EMR.

As the focus of research into the etiology of BE has shifted more and more towards molecular signaling, we are now in the process of unraveling the molecular mechanism underlying the pathogenesis of reflux esophagitis and BE. To put these newly gathered understandings into clinical practice, we need to target these genes and signaling pathways. This asks for enhanced methods to manipulate the genetic makeup of the esophageal mucosa. We therefore discuss potential genetic targets and strategies for treatment of BE and prevention of EAC.

CLINICAL:

ENDOSCOPIC TREATMENT OF HGD AND EARLY EAC

To produce guidelines for best clinical and cost effective management of HGD and early mucosal cancer in patients with BE, the BADCAT, a multidisciplinary group of 85 specialists from 13 countries, reviewed more than 11,000 published papers that compared endotherapy with surgery to surveillance or to no action.[47] Afterwards, 92 questions were asked, covering topics from pathology, medical therapy and endoscopy to surgery and patient support. There was a strong international consensus for the management of HGD. There were 2 statements with 100% agreement: “following endoscopic therapy for HGD, endoscopic follow-up is required” and “endoscopic resection specimens provide more reliable diagnostic samples than biopsy samples for HGD”. In contrast, statements concerning the prognosis of multifocal and unifocal HGD were much disputed, stressing that this is where further research is urgently needed.

ENDOSCOPIC RESECTION

In EMR a segment of the esophageal mucosa is removed down to the submucosa. It provides larger tissue specimens than biopsies and therefore provides better staging information. Previous studies show that EMR for HGD and early cancers in BE is a safe technique with high 5-year-survival rates. Pech *et al.* investigated efficacy and safety of EMR of early EAC in 953 patients in a prospective cohort study and followed them for a median period of 55 months.[48] The rate of long-term complete remission was 94.2% and showed that EMR was highly effective and safe procedure in patients with early EAC.

RFA is a technique where the esophageal mucosa is ablated with the help of balloon-electrode emitting radiofrequency energy. This technique generates a circumferential thermal injury with controlled depth. Previous studies suggested that RFA is highly effective at removing Barrett's mucosa and HGD. A recent prospective, sham-controlled randomized trial showed that RFA was a safe procedure with a high rate of durability and a low rate of disease progression.[49] 119 patients with LGD and HGD that received RFA were followed for at least 2 years. After 2 years follow-up, 94% of the subjects with LGD and 89% of subjects with HGD had a complete eradication of intestinal metaplasia (IM). 80% of ablated subjects remained free of intestinal metaplasia without further RFA after 3 years and the number of serious adverse events was small (3.4%). Another group showed that acid suppression with PPI plays an important role in esophageal squamous re-epithelialization following RFA therapy in patients with BE.[50]

Endoscopic submucosal dissection (ESD) is an advanced technique for treating early gastrointestinal metaplasia that, until now, has been mostly used in Japan. The technique involves the injection of fluid into the mucosa, cutting of the surrounding mucosa and dissection of the submucosa beneath the lesion. Providing an en bloc specimen it is suitable for pathological diagnosis and staging. A group of U.S. researchers[51] performed ESD in 19 patients with HGD or EAC and found it to be a feasible and safe procedure without larger complications. A Japanese study[52] described the long-term survival rates of patients with EGJ adenocarcinomas < 5 cm initially treated with endoscopic resection (ER). 53 patients with mucosal cancer of the EGJ, without lymph node or distant metastasis underwent EMR and were followed up to 9 years (or death) and outcomes compared to those of non-EGJ gastric cancers. 13 of the patients needed additional surgery. Complete resection rate (58%) was significantly lower in that for gastric cancers (76%), as well as diagnostic accuracy for the invasion depth of EGJ cancers (63%, vs. 85% for gastric cancers). In addition, there were 2 perforations and 2 postoperative bleedings which could be treated successfully by endoscopy. Overall 3-year and 5-year survival rates for all patients were 96% and 88%.

PRECLINICAL:

POTENTIAL THERAPEUTIC TARGET GENES: VEGF AND VITAMIN D RECEPTOR

Vascular endothelial growth factor (VEGF) is known to sustain angiogenesis and has recently been shown to exert pro-proliferative and pro-survival effects on cancer cells through binding to its receptors (VEGFR1 and VEGFR2). One study presented that VEGF signaling has pronounced effects on cell growth in transformed Barrett's epithelial cells, which are far more sensitive to those effects than non-transformed Barrett's epithelial cells, supporting a potential role for anti-VEGF therapies in the treatment of BE and EAC.[53]

Another interesting therapeutic target is Vitamin D, which has been shown to have several generic anti-carcinogenic effects such as suppressing cell proliferation, promoting cell differentiation and regulating apoptosis. Protective effects of high vitamin D status have been established in many cancers such as colorectal cancer, although there have been notable exceptions such as one study of pancreatic cancer[54] and one of esophageal squamous cell carcinoma.[55] An all-Ireland case-control study EAC cases had significantly higher vitamin D intakes compared with controls.[56] Controversely, at DDW a group presented that treatment with calcitrol, the active form of Vitamin D, markedly inhibited growth of EAC cells relative to controls. Only for OE33, a modest two fold induction of COX-2 expression was observed. Induced expression of CYP24A1, which initiates degradation of calcitriol, occurred in all three cell lines. Thus, even though growth inhibition of EAC cell lines advocates vitamin D as a potential therapeutic target, the induction of a negative feedback loop may be a mechanism for tumor cells to evade the effects of vitamin D.[57] Our group reported on the genetic variation in the VDR gene and its functional implications on esophagitis, BE, and EAC. Our study shows that a GT-haplotype near the conserved 1c-region of the VDR gene is associated with a twofold decreased susceptibility to esophagitis, BE, and EAC. We further show that this mutation causes an increase in GATA-1 binding to this regulatory region of the VDR gene, thereby inhibiting its transcription. These observations suggest that a decrease in VDR could have a beneficial effect on EAC (see Figure 1).[58]

POTENTIAL THERAPEUTIC TARGETING BY MICRORNAS

MicroRNAs (miRNAs) are small, naturally occurring RNAs that regulate multiple target genes by destabilizing mRNA or inhibiting translation. Regulating gene expression may be feasible through miRNAs. Recently, there has been a growing knowledge on miRNAs in BE and EAC.[59] At DDW, van Baal *et al.* elegantly showed from microarray data that specific miRNAs are overexpressed in human biopsies of BE tissue compared to squamous and adenocarcinoma. Focusing on genes with a more than two fold change in expression, miR-145 was discovered. It was reported

that miR-145 is higher expressed in BE than in normal squamous esophagus, however, its function in the esophagus is unknown. In stem cells, BMP4 expression is correlated with miR-145 expression, proposing an effect of miR-145 on BMP4 expression and its signal transduction pathway in the esophagus. In esophageal squamous epithelial cells, they demonstrated that miR-145 overexpression causes a decrease in proliferation 72 and 96 hours after transfection. miR-145 indirectly targeted BMP4 protein expression via specific reduction of transcription factor GATA6 expression. Downregulated BMP4 protein expression leads to decreased signaling as indicated by reduced Smad 1/5/8 phosphorylation and ID2 protein expression at 72 hours after transfection. This effect disappeared 96 hours after miR-145 transfection, indicating a negative feedback loop. These results demonstrate that miR-145 might well be involved in BE development by its effect on BMP4 signaling and encourage its putative role in chemoprevention of BE.[60] Additionally, a study observed an upregulation of protooncogenes c-Myc and Notch-1 with a corresponding decrease in tumor suppressor miRNAs let7a, miR-200a, and miR-144.[61] Together, these data suggests miRNAs as a new strategy for the development of novel anti-cancer treatments (see Figure 1).

ESOPHAGEAL SPECIFIC GENETIC MANIPULATION

One possible way of genetic manipulation presented at DDW, showed the use of nontraumatic PTFE catheter in the distal esophageal to deliver adenovirus to transduce specific genetic changes in the esophagus. In C57BL6 mice, by day 7, tissue undergoes pressure necrosis. 8-12 weeks after surgery, moderate reflux injury and tongues as well as islands of columnar mucosa surrounded by injured squamous epithelium. Using cre-recombinase adenoviral infection, Notch-GFP expression was specifically induced in esophageal squamous cells that started on day 3 and persisted up to 15 days. A subset of mice that had floxed constitutively active intracellular notch domain showed Notch activation by Cre-recombinase adenoviral infection.[62]

RESEARCH HIGHLIGHTS ON PREVENTION

At present, the main purpose of treating BE patients with PPI is to control their reflux symptoms. However, studies have suggested that these drugs may also prevent the development of EAC. Acid exposure might stimulate proliferation and inhibit apoptosis and therefore promote carcinogenesis in Barrett's mucosa.

PPI, NSAIDS & STATINS

Studies on nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase (COX) have suggested that these drugs might also protect against EAC.[63] COX-2 is highly expressed in the specialized intestinal metaplasia of BE and inhibition of COX-2 has been shown to have anti-proliferative and pro-apoptotic effects on EAC cell lines. Statins have a similar effect in vitro as will, by inhibiting proliferation and inducing apoptosis in EAC cells via inhibition of Ras farnesylation and inhibition of the ERK and AKT signaling pathways.[64] A recent study on the use of aspirin, NSAIDS and statins in BE patients showed a significant risk reduction of developing EAC.[65] Disadvantages of these drugs on the other hand are the cardiovascular risks and high cost of COX-2 selective NSAIDS and the gastrointestinal side effects of aspirin.

A multicenter prospective cohort study from the Netherlands followed 570 patients with BE over a median duration of 7.9 years.[66] Information about medication use was collected from patient interviews and pharmacy records. During the time of follow-up, 99% of all patients used a PPI; NSAIDS were prescribed in 70% and statins in 37%. 30% of patients used both statins and NSAIDs. There was a significant risk reduction of neoplastic progression in patients using NSAIDs (HR 0.51) and in those using statins (HR 0.36). Use of both NSAIDs and statins was associated with an additional reduction in risk (HR 0.19). A British study that investigated the effect of statins on the development of esophageal carcinoma in a large cohort of approximately 4 million people, found that the use of statins was negatively associated with the development of both, EAC and squamous cell carcinoma (OR 0.84).[67] The median length of statin use in this group was 3.7 years. Both NSAIDs and statins seem to have a protective effect and might be useful drugs if future studies can show that their protective effect outweighs side effects, but further elucidation of the mechanisms involved would help to tailor such chemopreventive strategies better to particular risk groups (see Figure 1).

FUTURE PERSPECTIVES

Patients with BE have a 30- to 125-fold increased risk of EAC relative to the general population. Only a small percentage of BE patients express GERD symptoms, and it is unknown why only a minority of patients with these symptoms develop BE. Accordingly, identifying subjects more prone to develop BE or EAC from the general population remains challenging. In predicting the risk of multifactorial disorders such as BE and EAC, identification of disease associated genetic makeup is essential. This highlights the need for genome-wide arrays as well as genetic association studies in esophageal disorders.[68]

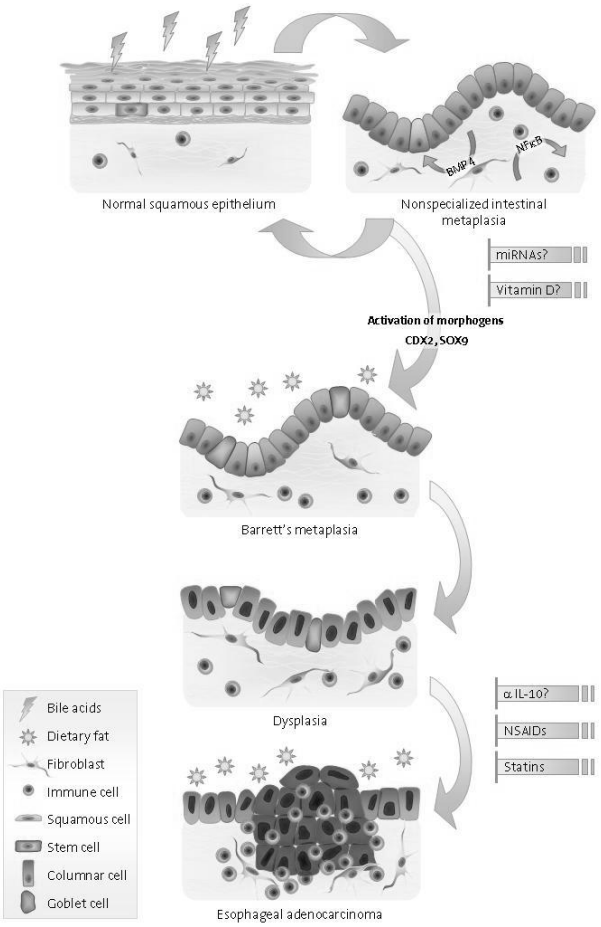

Figure 1. Schematic overview of the digest of DDW 2011 as summarized in this report.
In the first place, this figure portrays the continual transdifferentiation upon bile acid damage, which embodies stromal changes such as the activation of BMP4 and NF-κB pathway. Ensuant signaling to stem cells may reactivate morphogenes such as CDX2 and SOX9 and bring about the permanent switch to specialized Barrett's metaplasia. Secondly, new preventive strategies that were postulated at DDW using miRNAs or vitamin D are depicted, as well as possible sources of immunosuppressive and chemopreventive therapies at the stage of neoplasia.

The well-established increase in prevalence rates will have significant implications for health resource utilization and costs, due to the small but significant risk of developing esophageal adenocarcinoma. Endoscopic surveillance with the aim of detecting early lesions has been advocated. Endoscopic recognition and grading remains a significant to inform clinical decision. However, a truly cost-effective surveillance strategy remains to be determined. If we can find accurate blood-based biomarkers or genetic markers predictive of EAC, it may increase compliance with endoscopic surveillance and save some patients the procedure. Besides, a blood-based biomarker could reduce surveillance costs dramatically. To date, these are still unavailable for clinical application and require new prospective studies with long follow-up need to focus on biomarkers that predict risk and progression in BE patients.

The first step in moving towards the development of molecular treatment for clinical application should be finding accessible targets. Observations on BMP and CDX suggest a target pathway for developing molecular strategies to treat intestinal metaplasia and prevent development of the highly malignant EAC. The next step will be to selectively target the expression of genes in the esophagus. The use of miRNAs can than help to radically improve selective gene therapy in BE patients. Some miRNAs are presumed to have oncogenic effects by downregulating tumor suppressor genes, suggesting that inhibition of miRNAs is promising in developing future BE and EAC therapies for clinical practice.

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CHAPTER 3

EXPRESSION, LOCALIZATION AND POLYMORPHISMS OF THE NUCLEAR RECEPTOR PXR IN BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

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BMC gastroenterol 2011;11:108

ABSTRACT

Background and aim: The continuous exposure of esophageal epithelium to refluxate may induce ectopic expression of bile-responsive genes and contribute to the development of Barrett's esophagus (BE) and esophageal adenocarcinoma. In normal physiology of the gut and liver, the nuclear receptor Pregnane X Receptor (PXR) is an important factor in the detoxification of xenobiotics and bile acid homeostasis. This study aimed to investigate the expression and genetic variation of PXR in reflux esophagitis (RE), Barrett's esophagus (BE) and esophageal adenocarcinoma.

Methods: PXR mRNA levels and protein expression were determined in biopsies from patients with adenocarcinoma, BE, or RE, and healthy controls. Esophageal cell lines were stimulated with lithocholic acid and rifampicin. PXR polymorphisms 25385C/T, 7635A/G, and 8055C/T were genotyped in 249 BE patients, 233 RE patients, and 201 controls matched for age and gender.

Results: PXR mRNA levels were significantly higher in adenocarcinoma tissue and columnar Barrett's epithelium, compared to squamous epithelium of these BE patients ($P < 0.001$), and RE patients ($P = 0.003$). Immunohistochemical staining of PXR showed predominantly cytoplasmic expression in BE tissue, whereas nuclear expression was found in adenocarcinoma tissue. In cell lines, stimulation with lithocholic acid did not increase PXR mRNA levels, but did induce nuclear translocation of PXR protein. Genotyping of the PXR 7635A/G polymorphism revealed that the G allele was significantly more prevalent in BE than in RE or controls ($P = 0.037$).

Conclusions: PXR expresses in BE and adenocarcinoma tissue, and showed nuclear localization in adenocarcinoma tissue. Upon stimulation with lithocholic acid, PXR translocates to the nuclei of OE19 adenocarcinoma cells. Together with the observed association of a PXR polymorphism and BE, this data implies that PXR may have a function in prediction and treatment of esophageal disease.

INTRODUCTION

Persistent regurgitation of gastroduodenal contents into the lower esophagus causes mucosal injury manifested as reflux esophagitis (RE).[1,2] As a complication of chronic RE, a Barrett's esophagus (BE) can develop.[3,4] BE is defined as an acquired condition in which the stratified squamous epithelium of the lower esophagus is replaced by specialized intestinal epithelium.[5] It is the sole commonly recognized risk factor for the development of esophageal adenocarcinoma (EAC)[6,7] and has an increasing incidence in the Western world.[8] While the importance of acid and bile exposure in the development of BE is well established,[1,5,9,10] only a small percentage of BE patients will ultimately develop EAC. It remains largely unclear which factors control the rate of neoplastic progression in BE.[11] A growing body of evidence suggests that the intrinsic adaptive response to the toxic bile acids from the gastroduodenal contents is unable to prevent injury to the esophageal lining, thus suggesting a role for bile-induced signaling in the progression of BE.[12]

An important step in understanding the adaptive defense mechanism against toxic substances has been the identification and characterization of the nuclear pregnane X receptor (PXR).[13-16] PXR belongs to the nuclear receptor subfamily of ligand-activated transcription factors that play a key role in the regulation of biliary transport systems and enzymes that confer a protective role against toxic bile acids.[12] This group of nuclear receptors includes the constitutive androstrane receptor and the vitamin D receptor.[17,18] In humans, PXR is most abundantly found in the liver, the small intestine and the colon.[13,15,16,19] It is activated by a structurally diverse array of xenobiotics and endogenous compounds, including bile acids and steroid hormones.[13,17,18] Variability at the PXR genetic locus is therefore thought to be associated with pathophysiological changes in steroid, cholesterol or bile acid levels.[14] Polymorphisms in the PXR gene are associated with diseases such as inflammatory bowel disease and primary sclerosing cholangitis.[20,21] As these chronic inflammatory diseases are associated with aberrant bile acid metabolism, there may also be a link between PXR and BE.

The specific aim of this study was to explore the expression and distribution of PXR in BE and adenocarcinoma patients and analyze possible associations in the PXR gene with esophageal disease. We show that PXR expresses in tissue of BE and adenocarcinoma patients, and that it translocates to the nucleus in esophageal adenocarcinoma cells upon bile acid stimulation. In addition, a link between PXR polymorphisms and esophageal disease was found.

METHODS

HUMAN SPECIMENS

For immunohistochemistry, multiple biopsies of adenocarcinoma tissue (n = 19), columnar epithelium from BE patients without dysplasia (n = 28) and squamous epithelium from RE patients (n = 8) were taken at the same distance from the z-line. As healthy controls we included subjects that had no gastroesophageal reflux disease (GERD) symptoms or endoscopically detected aberrations of the esophagus (n = 3). The number of biopsies taken was approximately four per patient, and varied between one and eight biopsies. For each patient, all biopsy specimens were embedded in one single block of paraffin and were therefore stained and analyzed in one slide. Histological diagnosis was made by two experienced gastrointestinal pathologists (HD and HV). All patients had specialized intestinal metaplasia and were graded according to the most severe stage found. Cases on which agreement could not be reached or that were indefinite for dysplasia were excluded from this study.

Table 1 gives patient characteristics of the population used for analysis of PXR mRNA levels. mRNA levels were determined in a total of 119 esophageal samples, counting biopsies from 11 adenocarcinoma patients, duplicate biopsies of both the squamous and the columnar epithelium from BE patients (n = 21), squamous epithelium of RE patients (n = 7), and squamous epithelium of healthy controls (n = 5) without GERD symptoms or endoscopically detected aberrations of the esophagus. All BE patients had histologically confirmed intestinal metaplasia without high-grade dysplasia.

Table 1. Patient characteristics for PXR mRNA analysis

	RE (n = 7)	BE (n = 21)	EAC (n = 11)
Age, y (range)*	43 (21-60)	61 (34-78)	62 (42-73)
Male, (%)*	71	71	82
Type of epithelium	Sq	Sq, CE	tumor

RE: reflux esophagitis, BE: Barrett's esophagus, EAC: esophageal adenocarcinoma, Sq: squamous epithelium, CE: columnar epithelium *Groups did not differ significantly in gender. As expected, BE and EAC patients were somewhat older than RE patients

Characteristics of the group included in this study for genotyping are shown in Table 2. The total of 683 genetically unrelated Caucasians included 249 BE patients, 233 RE patients and 201 controls without any history of GERD symptoms, who all

Table 2. Patient characteristics per group for genotyping

	HC (n = 201)	RE (n = 233)	BE (n = 249)
Age, y (range)	57 (18-90)	54 (19-88)	61 (33-95)
Male, (%)	57	54	69
Length of BE segment, cm (SD)	NA	0	4.23 (2.39)

HC: healthy controls, RE: reflux esophagitis, BE: Barrett's esophagus, NA: not applicable

visited the endoscopy unit of the Erasmus MC-University Medical Center Rotterdam or the IJsselland Hospital in Capelle aan den IJssel between November 2002 and February 2005.[22] This study was approved by the institutional ethics review committees, and all patients gave informed consent before participating in the study.

CELL LINES

The human adenocarcinoma cell line OE19 and human squamous epithelial cell line HET1A were obtained from the ATCC. OE19 cells were grown RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mmol/l glutamine, 100 units/ml penicilline and streptomycin. HET1A cells were cultured in serum-free BRFF-EPM2 medium supplemented with 100 units/ml penicilline and streptomycin. Cells were maintained routinely at 37°C in 5% CO_2 humidified atmosphere. After a period of at least 24 h to allow cells to adhere to cover slips in 6-well plates (Greiner Bio-One) they were stimulated with 10 μM of rifampicine, 50 μM lithocholic acid (LCA), or 50 or 100 μM taurolithocholic acid (TLCA) for 24 h.

REAL-TIME PCR MRNA QUANTIFICATION FROM HUMAN ESOPHAGUS SAMPLES

Total RNA was extracted from tissue biopsies using TriReagent (Sigma, St Louis, MO) and purified using an RNeasy micro column kit (Qiagen, Hilden, CA). One-fortieth of a 1 μg cDNA synthesis reaction (iScript cDNA Synthesis Kit; Bio-Rad) was used in a 25 μl Real Time-PCR using SYBR GreenER (Invitrogen, Carlsbad, CA). The following primers were used for PXR gene amplification: 5'-ATGGCAGTGTCTGGAACTAC-3' and 5'- CAGTTGACACAGCTCGAAAG-3'. Duplicate samples were run three times in independent PCR runs and the average level of PXR was normalized to GAPDH using the ΔCt method.[23]

IMMUNOHISTOCHEMISTRY

Formalin fixed, paraffin embedded, five μm sections were mounted on glass slides. After deparaffinization in xylene and dehydration in alcohol, endogenous peroxidase was inactivated by incubation with 1% hydrogen peroxidase in methanol for 20 min.

Microwave pretreatment in glycin-HCl/EDTA buffer (50 mM Glycin, 10 mM EDTA, pH 3.5) was performed for 10 min. After treatment with 10% normal human plasma/10% goat serum to block non-specific antibody binding, sections were incubated overnight at 4°C with a rabbit anti-human PXR antibody (diluted 1:200, clone poly6169; Biolegend; San Diego, USA), followed by a biotin-labeled mouse anti-rabbit IgG (diluted 1:200; Dako, Glostrup, Danmark), and streptavidin-horseradish peroxidase (diluted 1:300, Dako) and visualized with diaminobenzidine. Nonspecific background controls were done by omitting the primary antibody and an isotype control was included. Samples of the terminal ileum served as a positive control. Sections were evaluated at a 200- and 400-fold magnification using light microscopy (Axioskop 20, Zeiss) by two independent observers (AW and KZ). At least 100 cells were counted in representative areas of longitudinally sectioned crypts in BE cases or high power fields in adenocarcinoma cases. For quantification only cases with nuclear protein expression were considered PXR positive, with cases evaluated as positive for PXR when more than 2% of counted cells showed nuclear positivity of PXR protein.

CONFOCAL MICROSCOPY

Cells cultured on coverslips were washed with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde for 10 min. After washing, cells were permeabilized with 0.2% Triton X100 for 20 min and then blocked with 5% goat serum and 5% normal human plasma in PBS with 5% BSA. Cells were incubated with mouse IgG or anti-hPXR antibody (1:200; Biolegend, San Diego, USA) at 4°C overnight and then probed with 1:200 dilution of goat anti-rabbit Alexafluor 594 (Invitrogen; Oregon, USA). Hoechst 33342 was used to stain nuclei. Coverslips were mounted onto glass slides with gelvatol and visualized under a Zeiss LSM 410 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany).

GENOTYPING

Genomic DNA was extracted from 5 ml of whole blood by a wizard genomic DNA purification kit (Promega, Madison, USA). We analyzed polymorphisms -25385C/T, 7635A/G, and 8055C/T as these should be informative for eight PXR polymorphisms and were observed by Zhang *et al* [14] to have an effect on PXR function in humans. Assay validation setup was performed by K-Biosciences (Herts, UK) before performing a double blind analysis of PXR SNPs with a competitive allele-specific PCR system using primers designed in flanking region of the SNP located at -25385; TGGTCATTTTTTGGCAATCCCAGGTT[C/T]TCTTTTCTACCTGTTTGCTCAATCG at 7635; AGGAGCCATCCTCCCTCTTCCTCTC[A/G]CCCCCAACTTCTGGATTATGGGATG and at 8055; GCTTGCTGAGAAGCTGCCCCTCCAT[C/T]CTGTTACCATCCACAGGTGGC TTCC of the PXR gene NR1I2.

STATISTICAL ANALYSES

The study was powered (80%) to allow detection of a 10% difference in genotype distribution of the PXR polymorphisms between the groups by performing Chi-square analysis. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated by risk estimate analysis. All statistical analyses were conducted using SPSS v11.0 (SPSS, Chicago, IL) and two-sided significance was taken as $P < 0.05$.

RESULTS

PXR GENE EXPRESSION IS ELEVATED IN BE AND ADENOCARCINOMA

PXR mRNA was determined by Real-Time PCR in a group of 44 subjects with different esophageal pathologies (Table 1). As shown in Figure 1A, levels of PXR mRNA were found consistently higher in columnar tissue compared to matching squamous tissue ($P < 0.001$), in which levels of PXR transcripts were barely detectable. Figure 1B shows interindividual differences in PXR expression between RE, squamous and columnar epithelium of BE, and EAC. The levels of PXR mRNA in the BE columnar epithelium were higher than in squamous epithelium of RE ($P = 0.003$, Figure 1B) and healthy controls ($P = 0.002$, data not shown). Also PXR gene expression in tissue of adenocarcinoma patients was significantly higher than in squamous samples from BE patients and healthy controls. Comparing RE with controls, only one patient showed a strong increase in PXR mRNA and thus overall difference in mRNA levels between these two groups did not reach statistic significance (Figure 1B).

PXR PROTEIN DISTRIBUTION IN BE AND ADENOCARCINOMA TISSUE

To test if the presence of PXR mRNA corresponded with the expression of PXR protein, esophageal biopsies of 39 patients were stained for PXR by immunohistochemistry. Figure 2 depicts representative stainings of PXR on esophageal biopsy specimens of healthy controls, and RE, BE, and adenocarcinoma patients. None of the normal squamous esophageal samples (n = 3) stained positive for PXR (Figure 2A). Also, no specific PXR signal was detected in RE samples (Figure 2B). In patients with histologically confirmed BE (n = 28), six cases of nuclear positivity were found (Figure 2C). In 17/19 adenocarcinoma patients, PXR expression was observed in the nuclei of cancer cells (Figure 2D). This was significantly higher compared to nuclear PXR expression in BE tissue ($P < 0.01$, Figure 2E).

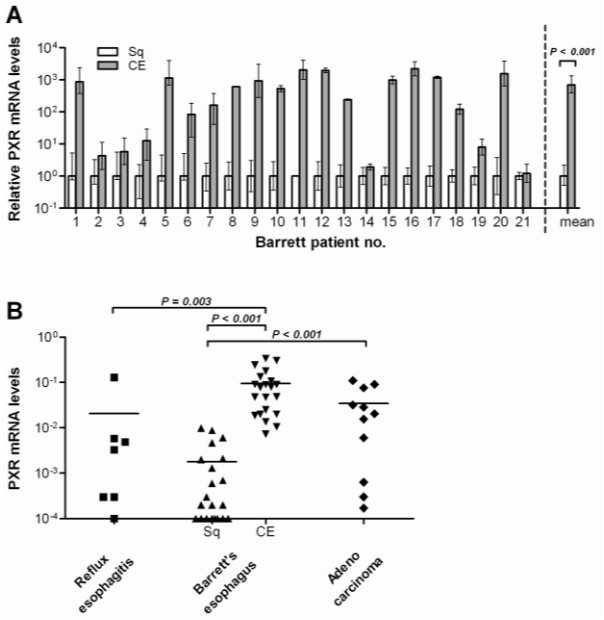

Figure 1. Relative mRNA levels of PXR in esophageal epithelium as determined by quantitative Real-Time PCR.
(A) PXR levels in columnar epithelium (CE) are normalized to adjacent squamous epithelium (Sq) using $2^{(-\Delta\Delta Ct)}$ method[23] and are plotted for each of 21 Barrett's esophagus (BE) patients. Error bars express a range which is a result of incorporating the standard deviation into the calculation. The mean of this population renders a strong significant increase of PXR mRNA levels in CE compared to Sq of BE patients ($P < 0.001$). (B) PXR mRNA levels are calculated using $2^{(-\Delta Ct)}$ to show interindividual differences in PXR expression in RE, BE, and EAC patients and plotted on a log scale. Levels in Sq from patients with reflux esophagitis, and patients with BE are lower than in CE derived from the esophagus of BE patients ($P = 0.003$ and $P < 0.001$ respectively). mRNA levels in tissue from adenocarcinoma patients did not differ statistically from CE of BE patients, but was significantly higher than all Sq tissues. The detection limit for this assay was 0.0001.

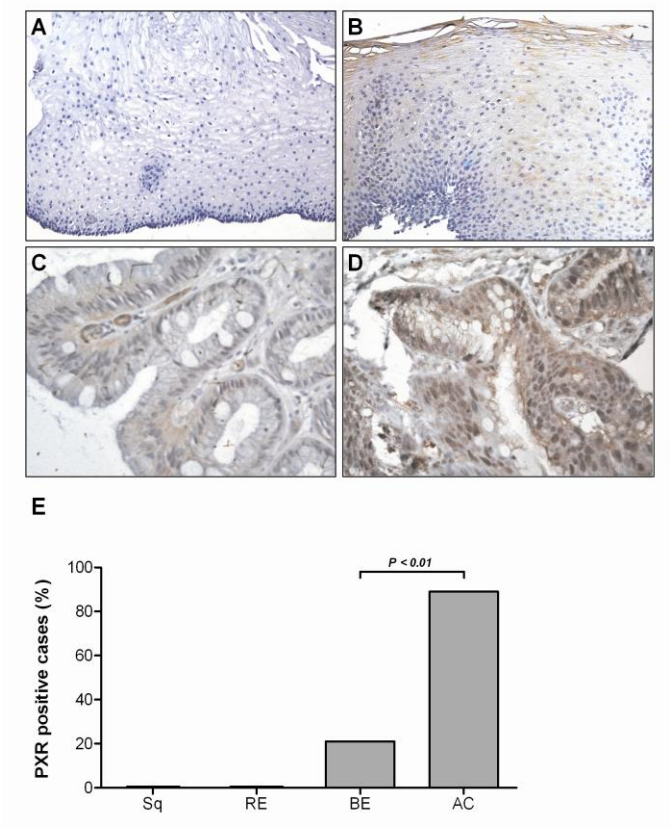

Figure 2. Result of PXR immuno-histochemical staining on esophageal biopsy specimens.
(A) Esophagus of healthy controls is lined by a stratified squamous epithelium and is negative for PXR (200x). (B) The esophageal mucosa of patients with reflux esophagitis is damaged and inflamed and demonstrates a weak signal for PXR (brown) in the cytoplasm of epithelium (200x). (C) Barrett's epithelium is characterized by a single layer of columnar epithelium with the presence of typical goblet cells. Cytoplasmic as well as some nuclear PXR expression is observed (400x). (D) Cells of adenoma tissue show high levels of nuclear PXR expression (400x). (E) Quantification showed that the percentage of cases with more than 2% PXR-positive nuclei was significantly higher in EAC than in BE ($P < 0.01$).

EXPOSURE TO BILE ACIDS DOES NOT AFFECT PXR MRNA LEVELS, BUT DOES INDUCE NUCLEAR TRANSLOCATION

PXR mRNA levels were analyzed in HET1A and OE19 cells upon stimulation with 50 or 100 μM TLCA. PXR levels in the OE19 adenocarcinoma cell line were higher than in the squamous epithelial HET1A cells (P = 0.02), but mRNA levels did not differ between unstimulated cells and cells stimulated with TLCA (Figure 3A). Figure 3B shows immunofluorescence of PXR in the nuclei of OE19 cells that were unstimulated, or stimulated with TLCA. Induction with rifampicine was taken as a positive control. More nuclear PXR staining was observed in cells stimulated with 10 μM rifampicine (data not shown) and 50 μM TLCA compared to unstimulated cells, with most intense staining observed in TLCA stimulated OE19 cells. In summary, exposure of adenocarcinoma cells to bile acids and xenobiotics appears to induce nuclear translocation of PXR independent of its gene levels.

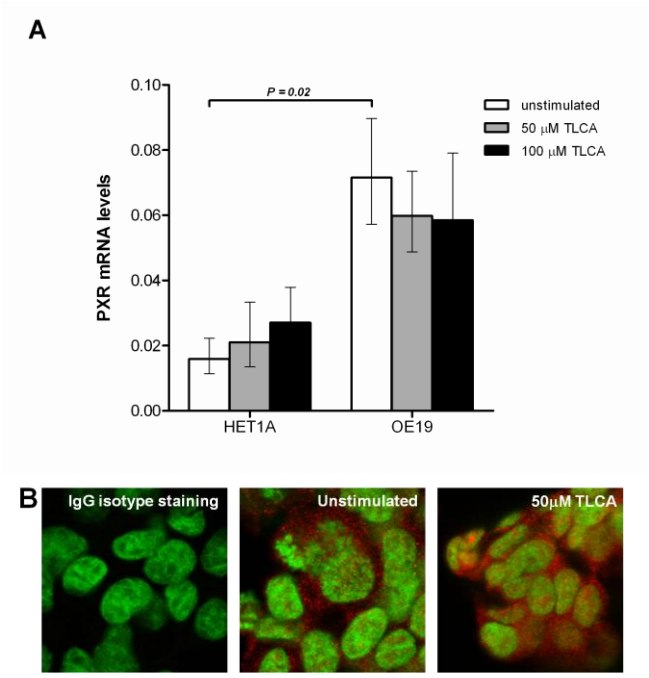

Figure 3. PXR mRNA levels and nuclear translocation of PXR protein in esophageal cell lines stimulated with bile acids.
(A) PXR mRNA levels are significantly higher in OE19 than in HET1A (P = 0.02). Bile stimulation with 50 μM or 100 μM of TLCA did not affect PXR mRNA levels compared to unstimulated conditions. (B) After immunofluorescent staining of PXR (red) and nuclei (green), localization in OE19 cells was visualized by a confocal laser microscope (1000x). In unstimulated cells, PXR was predominantly found in the cytoplasm. Upon 24 h of stimulation with 50 μM of TLCA, PXR translocation from the cytoplasm to the nuclei was observed.

PXR POLYMORPHISM 7635AG IS ASSOCIATED WITH BE

Polymorphisms at location 7635 and 8055 of the PXR gene have previously been found to be located in different linkage disequilibrium blocks and are thought to have an effect on PXR activity.[14] In our cohort the PXR gene polymorphisms were in Hardy-Weinberg Equilibrium. No significant association of SNP -25385C/T with BE or RE was found (P >0.5; data not shown). Allele frequencies of SNP 7635A/G and 8055C/T for patient and healthy control populations are listed in Table 3. Minor allele frequencies of these SNPs were in consensus with previous observations in European control cohorts.[14,24,25] Subjects carrying the SNP 7635G allele had an

increased risk of BE (OR 1.36, 95% CI 1.03-1.79). In comparing genotype distributions, an increase was demonstrated in the minor allele frequency among BE patients as compared with RE patients and healthy controls for both 7635A/G and 8055C/T. For SNP 7635A/G this trend was statistically significant (P = 0.037, Figure 4).

Table 3. Allele frequencies of PXR SNPs at locus 7635 and 8055

SNP		Allele frequency, no. (fraction)			HC *vs* RE	HC *vs* BE
locus	Allele	HC	RE	BE	OR (95% CI)	OR (95% CI)
7635	A	267 (0.674)	294 (0.636)	298 (0.603)	1.18 (0.89-1.57)	***1.36 (1.03-1.79)***
	G	129 (0.326)	168 (0.364)	196 (0.397)		
8055	C	321 (0.863)	381 (0.832)	397 (0.814)	1.27 (0.87-1.87)	1.44 (0.99-2.10)
	T	51 (0.137)	77 (0.168)	91 (0.186)		

HC: healthy controls, RE: reflux esophagitis, BE: Barrett's esophagus

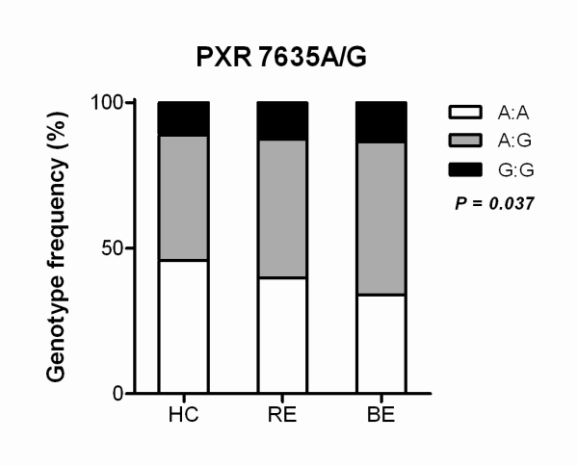

Figure 4. Genotype distributions of PXR polymorphisms across populations of healthy controls (HC), patients with reflux esophagitis (RE) and Barrett's esophagus (BE).
The distribution of AA (white), AG (gray) and GG (black) at locus 7635 of the PXR gene are depicted per patient group. As the pathologic condition of the esophagus progresses from healthy to RE to BE, prevalence of ancestral homozygous genotype decreases (P = 0.037).

DISCUSSION

The precise pathophysiological mechanisms causing BE is still unclear, but the combination of gastric acid and bile acids from the gastroduodenal reflux is commonly acknowledged as the key factor in the development of BE.[26] At low pH, bile acids are thought to cause esophageal mucosal injury, which has been substantiated both *in vitro* and in animal model systems.[27,28,29]

The NR1I family of orphan nuclear receptors are known to prevent toxic accumulations of xenobiotics within cells by regulating a broad range of cellular transporters.[17,30,31] The nuclear receptor PXR is a member of this family and functions in the enterohepatic organs as a detoxifier and regulator of bile acid homeostasis.[12-16] It can bind a variety of bile acids[32,33] and subsequently regulate the expression of a multitude proteins that transport bile acids across cell membranes.[34,35,36] These include the multidrug resistance (MDR)1 gene,[37,38] which encodes the efflux protein P-glycoprotein that removes xenobiotics from cells.[39] Other bile acid transporters that are induced by PXR include the multidrug resistance associated protein (MRP)2 and 3[40-42] and the organic anion transporting polypeptide (OATP)1 and 2.[42-44] From studies in mice it was concluded that the function of PXR is of particular importance when bile acid concentrations reach pathophysiologic levels.[33-45]

PXR expression is known in healthy liver and intestinal tract, but in cancer it has yet to be explored. Therefore, in this study we investigated the expression and significance of PXR in esophageal pathology. We did not detect PXR in normal squamous epithelium or in the squamous epithelium of RE patients. PXR did however express at both mRNA and protein level in columnar epithelium, and was significantly lower in adjacent squamous esophageal epithelium of the same patient. In samples from adenocarcinoma patients PXR was clearly observed the nucleus. PXR mRNA levels between BE and EAC do not differ, but nuclear PXR protein expression does increase in EAC. Perhaps, this is an effect of difference posttranscriptional modifications between the stages. It could also indicate a translocation from the cytoplasm to the nucleus occurring during progression from BE to EAC, as our studies showed translocation from cytoplasm to the nuclei of adenocarcinoma cells *in vitro* after stimulation with rifampicine or litholic acid. These processes and their significance to PXR function need to be further explored, and a first step in this could be Western blot analysis on subcellular fractions of BE and EAC cells.

Previous studies have suggested that PXR expression in cancer cells can interfere with the metabolism and responsiveness to chemotherapeutics, such as irinotecon and tamoxifen.[46,47] They suggest this drug resistance involves the metabolizing enzyme CYP3A4, one of the key target genes of PXR.[15] These effects on the metabolism of anticancer agents are especially important considering that PXR ligands include endogenous steroids and bile acids, as well as numerous environmental chemicals and dietary constituents. It has yet to be investigated whether higher levels of PXR in the esophagus also affects responsiveness to chemotherapy.

Given the relatively low rare allele frequency for SNP 8055C/T, our population size may have been insufficient to detect a statistically significant association. Validation

of our findings will require a well-characterized population from a multicenter study. Recent studies associate PXR polymorphisms with other pathogenic conditions of the gastrointestinal tract, such as inflammatory bowel disease[20] and primary sclerosing cholangitis.[21] Since associations with the two PXR SNPs in this study are in line with previous findings in IBD,[20] this draws attention on a possible link of the functional effect of these SNPs with chronic inflammation. It is well known that inflammation, through the activation of NF-κB pathway leads to a decrease of CAR, PXR and RXR-alpha expression and the expression of their target genes. In addition, it has recently been shown that the mutual repression between PXR and NF-κB signaling pathways provides a molecular mechanism linking xenobiotic metabolism and inflammation.[48]

Although it cannot be ruled out that the observed link between BE and PXR levels is not the cause but only the consequence of the metaplasia from squamous to intestinal-type mucosa, the link with PXR-activity associated SNPs suggest a active role of PXR in BE pathophysiology. Further research should focus on the biologic function of PXR in BE and EAC, especially because PXR protein expression was observed in only few nuclei in Barrett's epithelium whereas EAC tissue was abundant with PXR positive nuclei. Here, we chose LCA to study nuclear translocation as it is the endogenous ligand with the highest binding affinity for PXR. As supraphysiological levels of LCA were used to stimulate esophageal cells, further research will be required using extensive stimulation assays that mimick the *in vivo* situation by long-term repetitive stimulations with a mix of bile acids in physiologic concentrations as recently performed.[49] For a complex disease such as BE, development and validation of representative animal models will be of great value to investigate whether PXR plays a protective role in the development of BE or if it has a detrimental effect on neoplastic progression.

CONCLUSIONS

In summary, PXR which is normally not present in the squamous esophageal epithelium, is expressed highly in the columnar esophageal epithelium of BE patients and tumor tissue of EAC patients. At a protein level, this expression appears to be more nuclear in EAC than in BE. Upon stimulation with lithocholic acid, PXR translocates to the nuclei of OE19 adenocarcinoma cells. Together with the observed association of a PXR-activity associated SNPs and BE, this data implies that PXR may have a function in predicting progression and treatment of esophageal disease, though further studies are warranted to support this hypothesis.

ACKNOWLEDGEMENTS

The authors would like to thank our colleagues of the endoscopy units for their help, and their patients who volunteered to participate in this study. We thank Petra de Ruiter for her assistance in confocal microscopy and Hans van der Valk for expert pathologic grading.

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CHAPTER 4

DIFFERENTIAL EXPRESSION OF THE NUCLEAR RECEPTORS FXR AND PXR FOR GRADING DYSPLASIA IN PATIENTS WITH BARRETT'S ESOPHAGUS

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Histopathol 2011;58:246-53

ABSTRACT

Aim: To investigate expression of nuclear receptors FXR and PXR as a diagnostic tool to improve grading of dysplasia in Barrett's esophagus patients.

Methods: Immunostaining was analyzed on a total of 192 biopsy samples of 22 Barrett's patients with no dysplasia (ND), 17 with low grade dysplasia (LGD), 20 high grade dysplasia (HGD), and 24 with adenocarcinoma.

Results: Nuclear FXR expression was observed in 15/22 (68%) ND cases versus 5/60 (8%) patients with dysplasia or adenocarcinoma ($p<0.001$). FXR expression was highly specific for non-dysplastic tissue. Nuclear PXR was expressed in 16/20 (80%) HGD cases versus 2/16 (13%) LGD cases (PPV 89%). Upon examining adjacent tissue taken from HGD and adenocarcinoma patients, PXR expression was high in samples of all tissue types.

Conclusions: Nuclear receptors are differentially expressed during neoplastic progression with FXR-positivity being useful to distinguish ND from dysplasia and adenocarcinoma. PXR nuclear expression is able to separate HGD from LGD and ND. The combination of FXR and PXR appears to have diagnostic and possibly prognostic value as well, but future prospective studies are required to investigate their predictive power for dysplastic progression in Barrett's esophagus.

INTRODUCTION

Barrett's esophagus (BE) is an acquired condition of metaplasia in which the stratified squamous epithelium of the lower esophagus is replaced by specialized intestinal-like epithelium harbouring goblet cells.[1-3] The incidence of BE and esophageal adenocarcinoma have both increased rapidly since the 1970s and remain on the rise in the Western world.[4-7] Patients with BE have 30- to 125-fold increased risk of esophageal adenocarcinoma relative to the general population.[4, 8-10] Annual risk is estimated at a mere 0.2% to 2.1%.[10-12] Endoscopic surveillance is performed to detect early stages that lead to adenocarcinoma.[12, 13] The current marker for incipient malignancy in the esophagus is dysplasia, defined as the neoplastic proliferation within epithelial glands without affecting the basal membrane.

Neoplastic progression in BE follows a multistep sequence from intestinal metaplasia to low-grade dysplasia (LGD), high-grade dysplasia (HGD) until finally, adenocarcinoma.[2, 8, 14] At present it is unclear which factors control the rate of neoplastic progression in BE.[15] The presence and grade of dysplasia in random biopsies obtained during endoscopy are currently the best indicators of cancer risk in BE patients.[16] Patients with HGD have a high risk of developing adenocarcinoma warranting surgery or endoscopic mucosectomy.[17, 18] LGD on the other hand is more indolent.[19, 20] The initial diagnosis of LGD was expected to confer a two-fold increased risk of neoplastic progression as compared to BE without dysplasia.[19, 21-25] A major complication of using histology for grading dysplasia is that its assessment is subject to intraobserver and interobserver variation.[26, 27] The use of tissue biomarkers in addition to histology may improve the diagnosis of dysplasia and risk prediction in BE patients.[19, 24, 28] A considerable number of biomarkers have been suggested.[29] These include tumor cell markers, [30] and markers identifying genetic abnormalities,[31] DNA hypermethylation[32] and aberrant cell cycling.[33] Current biomarkers are not applied in clinical practice due to lack of reliability. This highlights the need for further research into new biomarkers to predict neoplastic progression.

It is known that the exposure of the esophageal lining to bile acids plays an important role in development and neoplastic progression of BE. Evidence suggests that detoxifying mechanisms in the esophagus fail to prevent injury to the esophageal mucosa.[34] This draws attention upon receptors involved in bile-induced signaling. Two nuclear receptors, the bile acid receptor Farnesoid X Receptor (FXR) and the xenobiotic sensor Pregnane X Receptor (PXR) have been identified.[35-37] They are abundantly expressed in the liver and intestine where they act as detoxifiers and regulate xenobiotic and bile acid homeostasis.[36, 38-41] In the human colon, the expression of FXR has been shown to progressively decrease as normal mucosa advances towards adenocarcinoma.[42] In the esophagus, overexpression of FXR in BE

compared to normal squamous epithelium and adenocarcinoma was reported.[43, 44] These studies further suggested that FXR expression contributes to the regulation of bile acid signaling and apoptosis in BE. We previously found that PXR mRNA levels in the esophagus are higher in Barrett's epithelium than in normal squamous epithelium, and that polymorphisms in the PXR gene are associated with the presence of BE (A. van de Winkel *et al*, submitted for publication). The aim of the present study was to investigate the expression of PXR and FXR during progression to dysplasia and cancer. Hereto, their presence and localisation was evaluated by immunohistochemistry in surveillance biopsies taken from BE patients with different stages of progression.

MATERIALS AND METHODS

PATIENT SAMPLES

Biopsy specimens from 83 BE patients (73% male; mean age 65 yrs, range 38-87) with different stages of progression were selected. Patients were assessed at the endoscopy unit of the Erasmus Medical Center Rotterdam and had endoscopically confirmed BE. From each patient, multiple biopsies were collected at the same distance from the z-line. The number of biopsies taken was approximately four per patient, and varied between one and eight biopsies. For each patient, all biopsy specimens were embedded in one single block of paraffin and were therefore stained and analyzed in one slide. Histologic diagnosis was made by two experienced gastrointestinal pathologists (HvD and HvdV). All patients had specialized intestinal metaplasia and were graded according to the most severe stage found. Cases on which agreement could not be reached or that were indefinite for dysplasia were excluded from this study. The four groups included BE without dysplasia (ND; n=22), LGD (n=17), HGD (n=20), or adenocarcinoma (n=24). Patient characteristics are given in Table 1. No significant differences in age or gender were found between the groups. This study was approved by the review board of the Erasmus Medical Center Rotterdam, the Netherlands.

Table 1 BE patient characteristics

	ND (n=22)	LGD (n=17)	HGD (n=20)	Adenocarcinoma (n=24)	Total (n=83)
Age, y (range)	63 (38-87)	68 (44-86)	67 (46-82)	62 (38-81)	65 (38-87)
Gender, %male	68	71	80	74	73

No significant differences were found between groups with respect to age or gender

IMMUNOHISTOCHEMICAL STAININGS

From the formalin-fixed, paraffin-embedded tissue blocks, sequential sections were sliced and mounted on adhesive slides (Starfrost, Berlin, Germany). After deparaffinization in xylene and dehydration in alcohol, endogenous peroxidase was inactivated by incubation with 1% hydrogen peroxidase in methanol for 20 min. Antigen retrieval was performed by boiling the sections for 10 min in Tris/EDTA pH 9.0 (for FXR) or in Glycine-HCl/EDTA pH 3.5 (for PXR). Sections were treated with 10% normal human plasma/10% goat serum to block non-specific staining. Anti human FXR/NR1H4 antibody (1:150; R&D Systems, Tokyo, Japan) was incubated for 1hr at RT, followed by polyclonal goat anti-mouse (1:200; Dako, Glostrup, Denmark). The PXR antibody (1:200; clone poly6169, Biolegend, San Diego) was incubated over night at 4°C and then 30 min at RT with a polyclonal goat anti-rabbit (1:200; Dako). After 45 min of incubation with streptavidin-HRP (Dako), FXR was visualized using 3-amino-9- ethylcarbazole and for PXR diaminobenzidine was used as a substrate. As a negative control the first antibody was omitted and an isotype control was included. Tissue of terminal ileum was taken as a positive control. Sections were evaluated at a 400-fold magnification using light microscopy (Axioskop 20, Zeiss) by two independent observers (AW, KZ). At least 100 cells were counted in representative areas of a longitudinally sectioned crypt or high power field. The scored percentages of positive nuclei were categorised as follows: no expression (<1%), mild (1-25%), moderate (26-50%) or high expression (>50%).[30, 45] Pictures were taken and analyzed using Nikon software (NisElements 2008, Tokyo, Japan).

STATISTICAL EVALUATION

The Mann-Whitney Test was applied for comparison of immunostaining results between groups. The Spearman correlation coefficient was used to evaluate significance of overall trends along the metaplasia-dysplasia-adenocarcinoma axis. To evaluate the utility of the markers, we constructed receiver operating characteristic (ROC) curves. All statistical analyses were conducted using SPSS v11.0 (SPSS, Chicago, IL) and 2-tailed significance was taken as $p<0.05$.

RESULTS

FXR NUCLEAR EXPRESSION DISTINGUISHES LGD FROM ND

Figure 1 shows FXR staining in ND (A), LGD (B), HGD (C) and adenocarcinoma (D). Nuclear FXR was typically expressed in ND patients (15/22; 68%), and was not or infrequently found in the tissue of LGD (3/17; 18%), HGD (0/19; 0%) and adenocarcinoma (2/24; 8%) patients ($p<0.001$ for overall trend; Figure 1E). Of FXR-positive ND cases almost 50% had moderate to high FXR expression levels (Figure

1F). The expression in LGD, HGD, and AC cases did not exceed the level of mild expression. FXR expression had a specificity of 82% in separating LGD from ND. This yielded a positive predictive value of 83%. ROC analysis confirmed that nuclear FXR expression was accurate in separating LGD from ND with an area under curve of 0.769 (p=0.004; Figure 2).

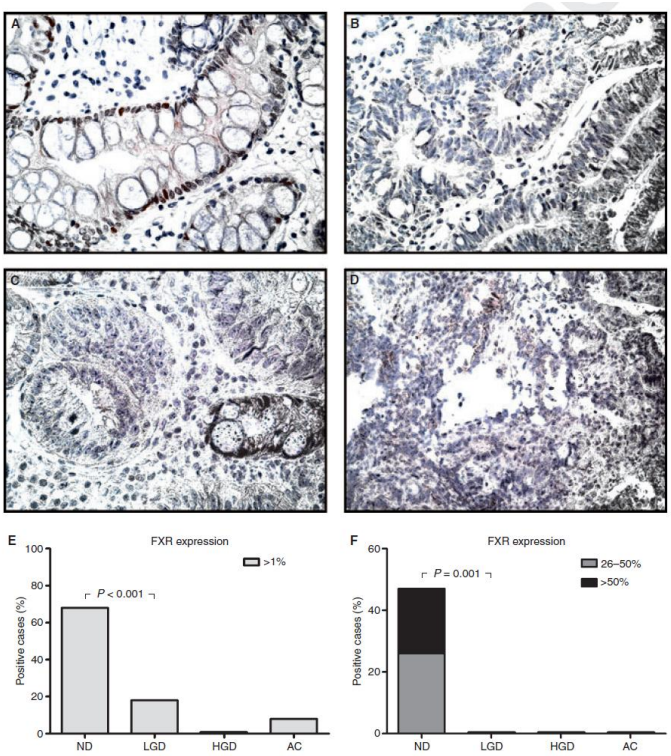

Figure 1. FXR staining in ND, LGD, HGD and adenocarcinoma patients.
Nuclear FXR was present in most cases of ND (A), but not in tissue of LGD (B), HGD (C) or adenocarcinoma patients (D). E) Quantification showed that significantly more cases of ND had nuclear FXR expression compared to LGD (p<0.001). F) The percentage of cases with moderate or high FXR expression levels was significantly higher in ND patients than in patients with more progressive stages of neoplasia (p=0.001).

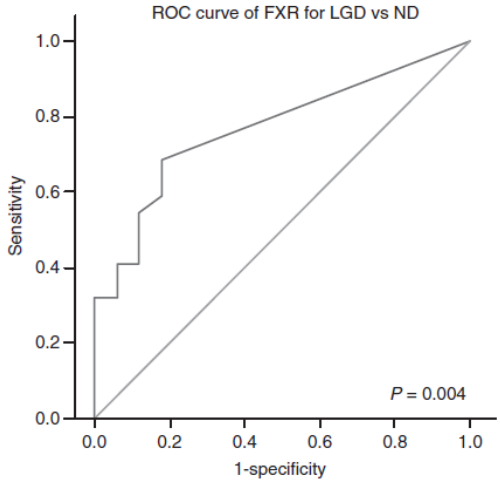

Figure 2. Receiver operating curve (ROC) of FXR nuclear expression comparing LGD and ND.
In the ROC-curve, 1% of FXR positive nuclei was taken as a cut-off to obtain the most sensitive and specific classification. The ROC-curve shows that the absence of nuclear FXR expression is an accurate tool to separate LGD from ND (p=0.004).

DIFFERENCES IN PXR NUCLEAR EXPRESSION BETWEEN HGD AND LGD

Figure 3 shows a representative PXR staining of ND (A), LGD (B), HGD (C), and adenocarcinoma (D) tissue. PXR expression was observed in only 4/21 (19%) ND and 2/16 (13%) LGD patients. In contrast, PXR-positive nuclei were present in tissue of 16/20 (80%) HGD and 17/22 (77%) adenocarcinoma patients ($p<0.001$; Figure 3E). The level of PXR expression was significantly higher in adenocarcinoma than HGD patients ($p=0.022$; Figure 3F). Grading LGD and HGD based on nuclear PXR expression complied in 89% of all cases with histologic grading. This conferred a specificity of 88%. ROC-curves yielded an area under curve of 0.852, indicating that PXR-positivity is a strong indicator of HGD (Figure 4).

NUCLEAR FXR AND PXR EXPRESSION IN TISSUE ADJACENT TO TISSUE OF HISTOLOGIC DIAGNOSIS

Nuclear FXR and PXR expression was examined in biopsy samples of the same patients taken directly adjacent to the biopsy on which histologic diagnosis was based. The percentage of patients that had FXR-positivity in adjacent tissue is shown

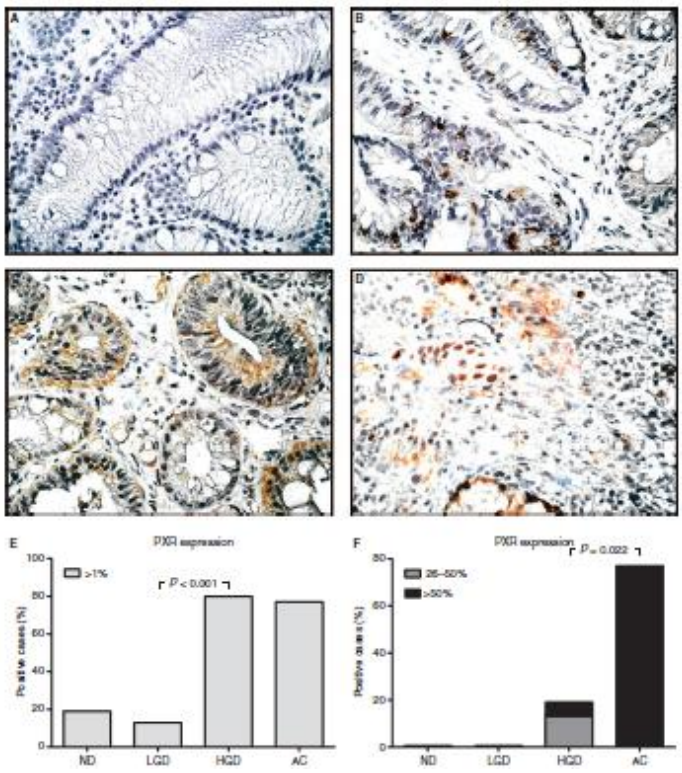

Figure 3. PXR staining in ND, LGD, HGD and adenocarcinoma patients.
Nuclear PXR was not expressed in ND (A) or LGD (B) tissue, but was observed in HGD (C) and adenocarcinoma (D) tissue. E) Quantification showed that significantly more cases of HGD had nuclear PXR expression compared to LGD ($p<0.001$). F) High PXR expression levels were found more often in adenocarcinoma (AC) than in HGD patients ($p=0.022$).

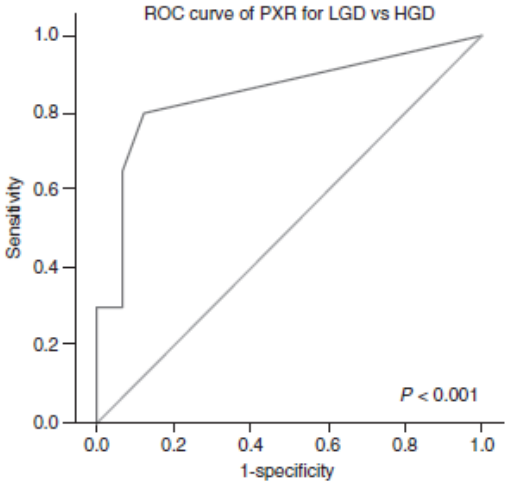

Figure 4. Receiver operating curve (ROC) depicts the accuracy of PXR nuclear expression as a marker for dysplasia by evaluating its sensitivity and specificity. Taking 1% of PXR positive nuclei as a cut-off rendered the most sensitive and specific classification of HGD from LGD. The AUC for this criterion was 0.852 ($p<0.001$).

in Figure 5A. In cases where adjacent normal squamous biopsy specimen was available, analysis showed FXR-positive nuclei in 38% of LGD, 38% of HGD and 67% of adenocarcinoma patients. Adjacent ND tissue was positive for nuclear FXR expression in 80% of LGD, 62% of HGD and 75% of adenocarcinoma cases (Figure 5A). PXR expression was completely absent in the adjacent normal squamous tissue of ND and LGD patients (Figure 5B). In adjacent ND tissues from LGD patients only 1/9 (11%) expressed PXR. In HGD patients analyzed cases did express PXR in the adjacent squamous tissue (4/8; 50%) and in the nearby ND tissue (4/7; 57%). In adenocarcinoma patients high PXR expression was found in adjacent normal squamous (12/14; 86%), ND (8/9; 89%), and in HGD (8/13; 62%) tissue (Figure 5B).

Overall these data show that FXR expression significantly differs between areas of dysplasia and adjacent ND and normal squamous tissue, whereas PXR expression is similar in all tissue samples of an individual patient.

DECISION CHART BASED ON FXR AND PXR DISTRIBUTION

Figure 6 shows a decision chart for using PXR and FXR as a diagnostic tool. FXR positive cases are likely to be ND. In the FXR-negative cases, PXR-positivity in any of the tissue is an indication for HGD or adenocarcinoma. Cases that have no FXR or PXR expression are classified as LGD patients. This decision chart can be used in addition to histology for classifying disease progression in BE patients. It offers potential clinical value in improving the accuracy of dysplasia grading especially by addressing the issue of sampling error.

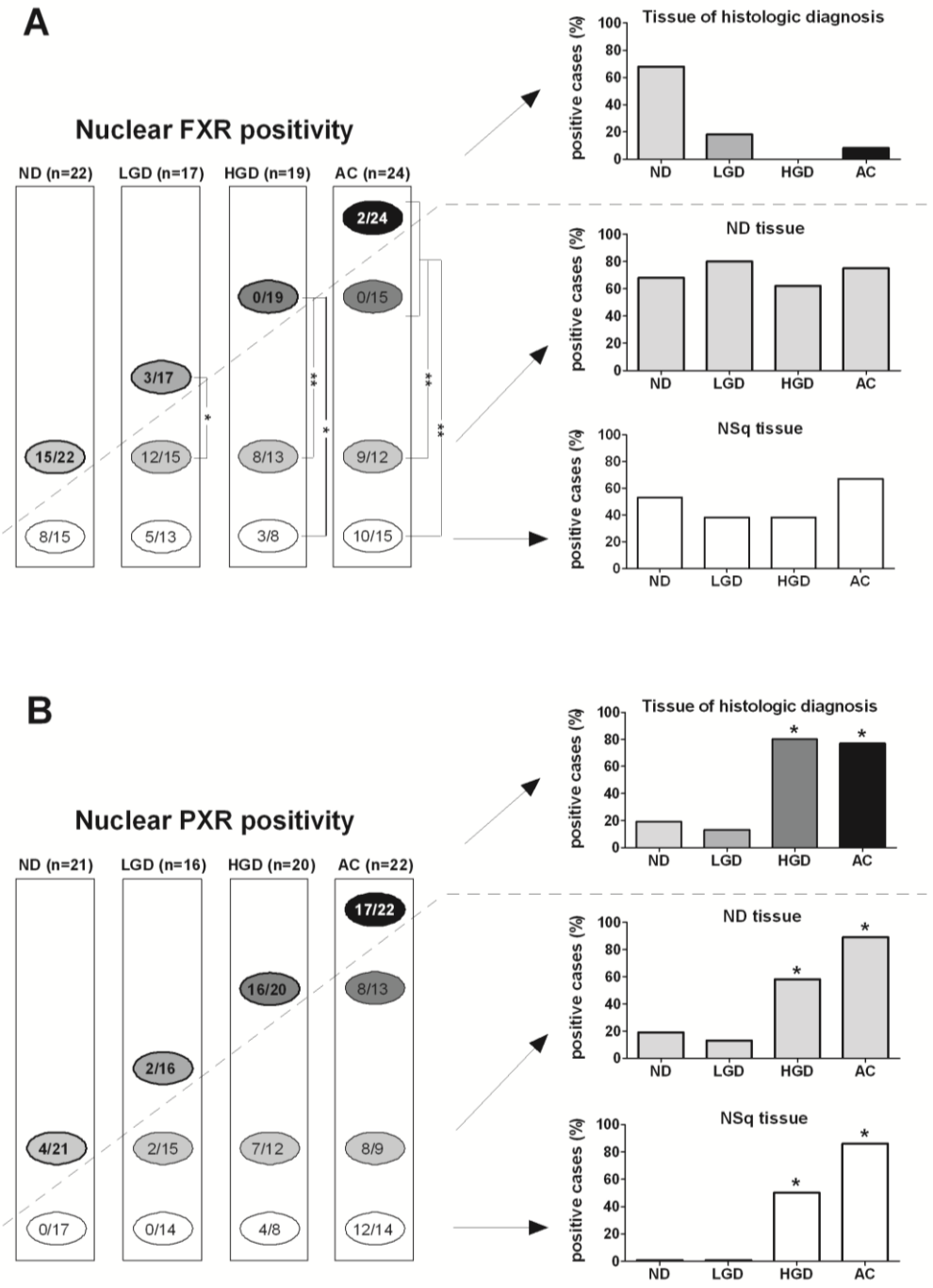

Figure 5. FXR and PXR expression in adjacent tissue to the biopsy of histologic diagnosis. A) The left panel shows FXR nuclear staining (positive samples/total samples) in biopsy specimen adjacent to the specimen of histological diagnosis (below dotted line). Bar graphs on the right show the percentage of FXR-positivity for adjacent normal squamous (NSq) tissue and adjacent ND tissue for each patient group. Though loss of nuclear FXR was observed in diagnostic specimens of LGD, HGD and AC patients, expression remained significantly higher in adjacent NSq and ND tissue (* $p<0.05$, $p<0.01$). The FXR-positivity in NSq and ND tissues did not differ between patient groups. B) The left panel shows PXR nuclear staining (positive samples/total samples) in biopsy specimen adjacent to the specimen of histological diagnosis (below dotted line). Bar graphs on the right show the percentage of PXR-positivity for adjacent NSq tissue and adjacent ND tissue for each patient category. Nuclear PXR expression in HGD and AC patients was also found elevated in adjacent NSq and ND tissue. The percentage of PXR-positivity in NSq and ND tissue of HGD and AC patients was significantly higher than in these tissue types found in LGD and ND patients (* $p<0.05$).

DISCUSSION

In this study we have investigated the expression of FXR and PXR in different stages of neoplastic progression in BE and their value as potential biomarkers. Nuclear expression of FXR was clearly present in 68% of ND cases, but detected in only few patients with dysplasia or adenocarcinoma. In contrast, nuclear PXR was specifically expressed in esophageal tissue of patients with HGD and adenocarcinoma, but not in patients with ND or LGD. Staining for PXR allowed an appropriate distinction between LGD and HGD in 83% of all cases, taking histology as a gold standard. As histology experiences difficulty in diagnosing dysplasia, the combined use of FXR and PXR could contribute to an accurate and reliable distinction between stages of dysplasia. This improvement is especially important when considering enhancement of risk stratification and cost-effectiveness in endoscopic surveillance strategies.

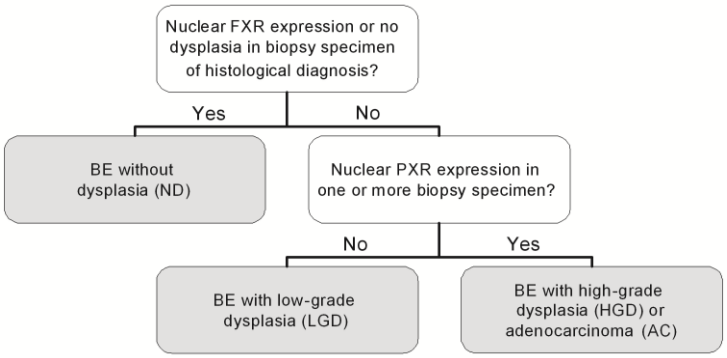

Figure 6. Decision chart combining FXR and PXR results as tool for histologic grading.
FXR positivity in tissue of histologic diagnosis is indicative for ND. Of the FXR negative cases, PXR positivity in any of the specimen taken during biopsy is an indication for HGD or adenocarcinoma whereas PXR negative cases are likely to be LGD patients.

In FXR-negative dysplasia and adenocarcinoma patients surrounding ND and normal squamous tissue was found positive for FXR. This underlines a highly grade and tissue specific regulation. The observed loss of nuclear FXR suggests it is inactivated in dysplastic and adenocarcinoma tissue. This can be accounted to either hypermethylation of the gene or mutational events.

Nuclear PXR was absent in all tissue types of ND and LGD patients. In HGD and adenocarcinoma patients PXR was highly expressed in all tissues with different stages of progression. Considering this data it appears that PXR is the result of a field effect of the adenocarcinoma. Therefore, the six ND and LGD patients that expressed PXR may actually be HGD cases that were missed because of sampling error. PXR expression could also address the issue of diagnosing those cases classified by pathologists as indefinite for dysplasia.

In a recent retrospective multicenter study,[46] investigators demonstrated that a model of eight methylation biomarkers predicted 50% of progressors to HGD and adenocarcinoma that would not have been diagnosed earlier without using these biomarkers. Based on our findings it is encouraging to evaluate FXR and PXR expression as a diagnostic tool. New prospective studies with long follow-up need to focus on risk prediction of FXR and PXR in BE patients.

In conclusion, FXR is expressed in the nuclei of ND tissue, but expression is lost during progression to dysplasia and cancer. Nuclear expression of PXR appears to be a field effect. It is high in all esophageal tissue of HGD and adenocarcinoma patients and is nowhere present in LGD and ND patients. The combination of FXR and PXR may prove valuable as a diagnostic tool and future studies are encouraged to investigate their role and predictive power in BE patients.

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CHAPTER 5

A VITAMIN D RECEPTOR GENE POLYMORPHISM THAT PROVOKES GATA-MEDIATED SUPPRESSION OF ESOPHAGEAL VDR EXPRESSION AND CONCOMITANT RISK REDUCTION FOR BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

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ABSTRACT

Background and aim: Epidemiological studies indicate that vitamin D exerts a protective effect with respect to the development of various solid cancers, including colon cancer, but overall survival benefits are disappointing and identification of cohorts most likely to benefit from vitamin D therapy represents an important goal in personalized chemopreventive medicine. In addition, recent concerns have been raised regarding the potential deleterious role of high vitamin D on the development of esophageal adenocarcinoma (EAC) or its precursor Barrett's esophagus (BE). The aim of this study is to investigate genetic variation of vitamin D receptor (VDR) in relation to esophageal expression and risk of contracting BE or EAC.

Methods: Fifteen haplotype tagging SNPs of the VDR gene were analyzed in 708 patients and 202 healthy controls and an independent BE replication cohort of 150 patients. Regulation VDR gene expression was investigated by immunohistochemistry, quantitative RT-PCR and gel shift assays.

Results: VDR expression was increased in the progression from squamous epithelium to BE. An 1633G/1453T haplotype was identified in a highly conserved parts of the 5´regulatory region of the VDR gene, which acts as a functional GATA transcription factor binding site and was associated with a significant reduced VDR expression in BE tissue. Moreover, this VDR haplotype was associated with an approximately two-fold reduced risk of contracting BE (OR 0.44; 95%CI 0.23-0.85) or EAC (OR 0.50; 95%CI 0.27-0.96) in two independent cohorts of patients.

Conclusion: We identified a novel functional VDR haplotype associated with reduced esophageal VDR expression and protection against acquiring BE or EAC. These polymorphisms may be useful for personalized chemopreventive medicine in identifying individuals likely to most benefit from vitamin D therapy.

INTRODUCTION

Incidence of esophageal adenocarcinoma (EAC) in Western Europe and North America has been on the upward trend for many decades. Despite advances made with respect to its treatment, EAC has a poor prognosis, and is the cancer with the fastest rising incidence of all cancer types. EAC often arises within Barrett's esophagus (BE), a metaplastic condition of the distal esophagus, in which through long-standing gastro-esophageal reflux disease, the normal squamous epithelium is replaced by columnar epithelium, morphologically having gastric, intestinal, or pancreatic qualities. Thus BE is considered a premalignant condition, associated with a 30- to 125-times increased risk for developing EAC compared to the general population.[1, 2] In the western countries, the prevalence of BE has been increasing dramatically since the 1970s, which explains the increasing incidence of EAC, but the risk factors contributing to this increase in BE remain less well-understood. BE is likely caused by a combination of genetics and environmental factors,[3] but few studies have examined the exact association with diet. An inverse association with increased intake of fruit, vegetables, and anti-oxidants has been reported, however, this association was not consistent across studies reviewed by De Ceglie et al.[4]

Vitamins and anti-oxidants are believed to be the key components of our daily diet with anti-carcinogenic action, including vitamin D. Vitamin D, actually a micronutrient rather than a vitamin, is the precursor to the steroid hormone calcitriol. It is obtained from dietary sources, but can also be produced endogenously under the influence of solar ultraviolet-B radiation.[5, 6] Its main action lies in normal development and mineralization of a healthy skeleton. With the discovery of the vitamin D receptor (VDR) in a variety of tissue that are not involved in calcium or phosphate metabolism, and considerable evidence in scientific literature citing an inverse epidemiological relationship between vitamin D and the incidence of several cancers,[7] another important role for vitamin D has emerged, namely in cancer prevention. Since then, a number of immunomodulatory and anti-tumor actions have been described.[8] An analysis of epidemiological data revealed that a serum 25-hydroxy vitamin D level of at least 130 nmol/l reduces incidence of breast cancer with 50% compared to low levels of 25 nmol/l.[9, 10]

The protective properties of vitamin D have been most extensively studied for colorectal cancer. Some meta-analysis have demonstrated consistent protective effects of high vitamin D status and in association with colorectal cancer and adenoma risk in particular[11, 12] Another meta-analysis of nine cohort studies concluded increasing serum levels of 25-hydroxyvitamin D (25(OH)D) are associated with reduced risk for colorectal cancer,[13] though confounding by physical activity and body mass index (inverse relation) has to be taken into consideration.[14, 15, 16, 17] To date, the main randomized controlled trial, the Women's Health Initiative, found that calcium and vitamin D supplementation had no effect on the incidence of

colorectal cancer compared to the placebo arm after a follow-up period (average of 7 years).[18] Thus, clinical trials addressing the potential usefulness of vitamin D supplementation have to date been somewhat disappointing and the role of vitamin D needs to be examined more extensively. Additionally, the hypothesised protective effects of vitamin D in cancer prevention may not be universal. There have been notable exceptions to this protective character as reported for serum 25-hydroxy vitamin D levels and prostate cancer risks,[19, 20] as well as risks of esophageal squamous cell carcinoma and its precursor, squamous dysplasia of the esophagus, in a Chinese population.[21, 22] Other epidemiological findings observe an increased prevalence of esophageal cancer in individuals with serum levels >75 nmol/L oral cavity and esophagus,[23, 24] as well as observed increased risk for developing esophageal cancer following dietary exposure to vitamin D or increased exposure to sunlight.[23, 24, 25] In addition, a significant direct association was observed between the highest tertile versus the lowest tertile of vitamin D intake and odds of EAC, even after adjustment for confounders, but did not find this correlation in pre-malignant stages of reflux esophagitis and BE.[26]

Factors that govern differences between population cohorts benefitting from vitamin D-mediated chemoprevention may lie in differential expression and/or activity of enzymes responsible for local activation and degradation of vitamin D, or variations in the expression or signaling of the VDR itself.

The aim of this study is to investigate the consequences of genetic variations in the VDR gene for esophageal VDR expression and development of BE, erosive esophagitis and EAC. Here we report the presence of a 1453C>T polymorphism in a highly conserved part of the 5´ non-coding regulatory region of the VDR gene that results in the creation of an apparently canonical GATA binding site in the VDR promoter which is associated with reduced VDR expression in the esophagus. Moreover, this polymorphism was associated with a decreased susceptibility to esophageal disease. Potential implications for vitamin D based chemoprevention will be discussed.

MATERIALS AND METHODS

HUMAN PATIENT AND HEALTHY CONTROLS

The association of VDR genotype with esophageal disease was analyzed in a group of 708 patients with RE, BE or EAC who visited the endoscopy unit of the Erasmus Medical Center Rotterdam or the IJsselland Hospital in Capelle aan den IJssel between November 2002 and February 2005.[27] Additionally, subjects visiting a general practitioner during this period for symptoms unrelated to and without any

previous symptoms of GERD were asked to participate and served as healthy controls (n=202). Patient characteristics are given in Table 1.

Table 1. Patient characteristics

	HC	RE	BE	Repl BE	EAC
Number of subjects	202	307	260	150	141
Mean age, y (range)	57 (18-90)	55 (19-88)	61 (33-95)	59 (30-87)	63 (38-87)
Male, %	57	56	69	89	82

HC: healthy controls, RE: reflux esophagitis, BE: Barrett's esophagus, Repl BE: BE replication cohort, EAC: esophageal adenocarcinoma. As expected, BE and EAC patients were somewhat older than RE patients

Subjects included in the RE population had endoscopically RE (n=307), which was graded according to the Los Angeles (LA)-classification.[28] Patients were diagnosed endoscopically with BE (n=260) defined as columnar lined segment in the esophagus of >2 cm in length with specialized intestinal metaplasia found histologically. The length of the columnar lined segment was determined endoscopically by measuring the distance between the squamocolumnar junction (the location at which the light-pink mucosa of the squamous-lined esophagus joined the red mucosa of the columnar lined epithelium) and the lower esophageal sphincter. Endoscopic diagnosis of adenocarcinoma (n=141) was confirmed by pathologic assessment of the histology of biopsies. An independent BE replication cohort (n=150) was collected from the Amsterdam Medical Center. This study was approved by the institutional ethics review committees, and all patients gave informed consent before participating in the study.

GENOTYPING

Genomic DNA was extracted from 5 ml of whole blood by a wizard genomic DNA purification kit (Promega, Madison, USA). Fifteen haplotype tagging SNPs (htSNPs) across the whole VDR gene for Caucasian population were genotyped with the use of the high-throughput TaqMan allelic discrimination assays. A random 5% of samples were independently repeated to confirm genotyping results.

REAL-TIME PCR mRNA QUANTIFICATION FROM HUMAN ESOPHAGUS SAMPLES

Total RNA was extracted from tissue biopsies using TriReagent (Sigma, St Louis, MO) and purified using an RNeasy micro column kit (Qiagen, Hilden, CA). One-fortieth of a 1 µg cDNA synthesis reaction (iScript cDNA Synthesis Kit; Bio-Rad) was used in a 25 µl Real Time-PCR using SYBR GreenER (Invitrogen, Carlsbad, CA). The following primers were used for VDR gene amplification: 5'-CCGCATCACCAAGGACAAC-3' and 5'- GCTCCCTCCACCATCATTCAC-3'. Duplicate

samples were run three times in independent PCR-runs and the average level of VDR was normalized to RNA polymerase II and GAPDH using the ΔCt method.[29]

IMMUNOHISTOCHEMICAL STAINING

From the formalin-fixed, paraffin-embedded tissue, five-µm tissue sections were sliced and mounted on adhesive slides (Starfrost, Berlin, Germany). After deparaffinization in xylene and dehydration in alcohol, endogenous peroxidase was inactivated by incubation with 0.5% hydrogen peroxidase in methanol for 15 min. Antigen retrieval was performed by boiling the sections for 10 minutes in 10 mM Citric acid monohydrate buffer, pH 6.0. Sections were blocked with 5% bovine serum albumine to block non-specific staining. Anti-VDR monoclonal antibody (1:200; clone 9A7, Affinity Bioreagents, Golden, USA) was incubate for 1 hour at RT, followed by polyclonal biotin labelled goat anti-rat (1:500; Dako, Denmark). After 45 min of incubation with streptavidin-HRP (1:300; Dako, Denmark), VDR was visualized using 3-amino-9- ethylcarbazole as a substrate and a hematoxillin counterstaining. Sections were evaluated using light microscopy (Axioskop 20, Zeiss) and pictures were taken and analyzed using Nikon software (NisElements 2008).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Oligonucleotides used in electrophoretic mobility shift assay (EMSA) and supershift assays were 5'-CCAGGGTGGTTGTCTACCTGGATGTCACCTCTGACCTCTG-3' and 5'-CCAGGGTGGTTGTCTATCTGGATGTCACCTCTGACCTCTG-3'. Probes were 5´ end-labeled with [γ-^{32}P]ATP. Nuclear extracts were prepared from MEL cells according to the methods used by Papachatzopoulou and coworkers.[30] For EMSA experiments, 2.5 µg of nuclear extract prepared from MEL cells were incubated for 30 min at 37°C with 2 ng of 32P-labeled or unlabeled VDR oligonucleotide probe in a binding buffer consisting of 50 mM Tris pH 8.0, 250 mM NaCl, 5 mM dithiothreitol, 5 mM ethylenediaminetetraacetic acid and 50% glycerol in a total volume of 25 µl. In competition assays, 100-fold molar excess of unlabeled competitor was included in the binding reaction. For supershift assays, 2 µg of GATA-1 (N6)X mouse monoclonal antibody (Santa Cruz Biotechnology, San Diego, CA, USA, sc-265 X) were added to reaction mixtures 30 min before addition of the nuclear extract. The protein-DNA complexes were separated from free probe by electrophoresis through a nondenaturing polyacrylamide gel and visualized on a orthochromatic film (Super HR-U30, Fuji Film, Tokyo, Japan) and developed using Fuji medical film processor Model FPM 100A (Tokyo, Japan).

STATISTICAL ANALYSES

Genotype distribution was tested for Hardy-Weinberg equilibrium. The study was powered (80%) to allow detection of a 10% difference in genotype distribution of the VDR haplotypes between the groups by performing Pearson Chi-square analysis. Odds ratio and 95% confidence interval (95% CI) were calculated by risk estimate analysis. Logistic regression analysis was applied to establish dose-allele models. All statistical analyses were conducted using SPSS v11.0 (SPSS, Chicago, IL) and 2-tailed significance was taken as $p<0.05$.

RESULTS

THE TRANSFORMATION OF NORMAL SQUAMOUS EPITHELIUM TO BE IS ACCOMPANIED BY INCREASED VDR EXPRESSION

Epidemiological studies led to the suggestion that vitamin D promotes EAC, but the molecular basis of this effect is unclear. To this end, we investigated VDR expression in a cohort of 25 patients, comparing normal squamous epithelium to BE in the same patients by qPCR. In the majority of patients, transformation to BE correlated with a strong increase in VDR mRNA expression (Figure 1A; $p<0.002$). This was also reflected in higher levels of VDR protein in the Barrett's segment, especially in the epithelial compartment, as evident from immunohistochemical staining of squamous and Barrett's biopsies from the same patient (Figure 1B). As shown in Figure 1C, in most BE tissue the VDR protein had a nuclear localization, suggesting activation of the receptor. Thus the progression of normal squamous epithelium to Barrett's epithelium is associated with substantial upregulation of VDR expression.

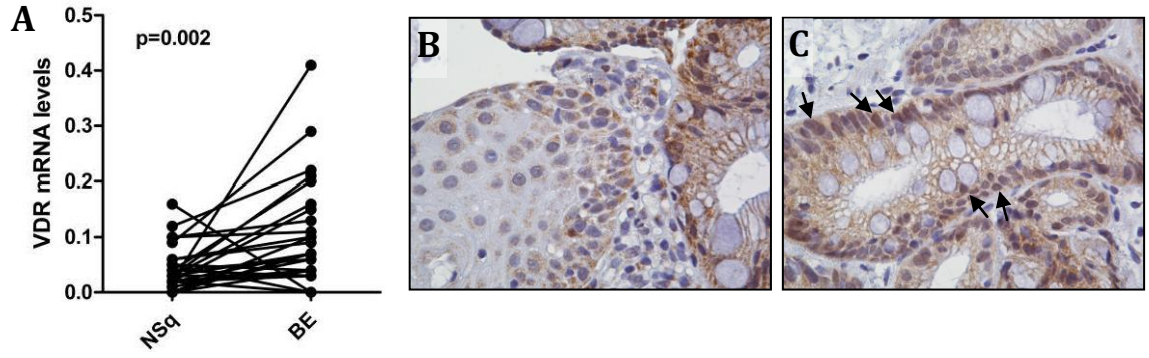
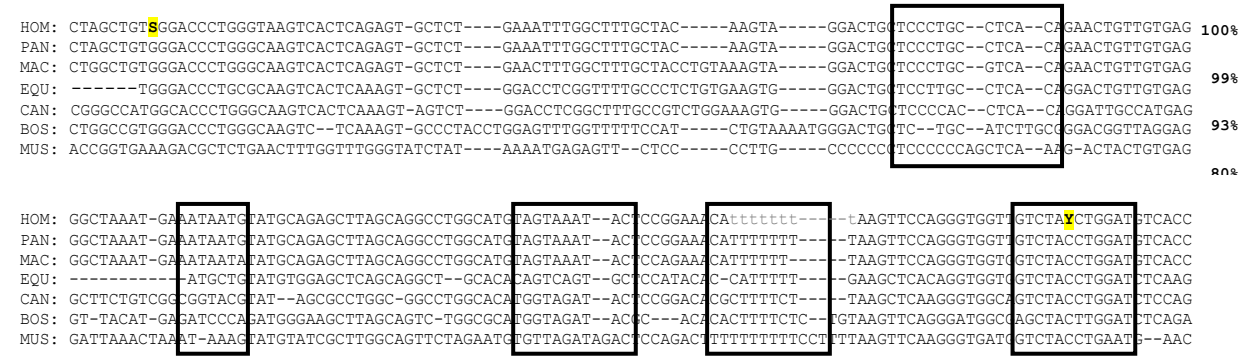


Figure 1. Esophageal expression of VDR in BE patients. A) Relative mRNA levels of VDR in paired squamous and columnar epithelium samples of BE patients. In 19 of 23 patients VDR mRNA was highest in columnar epithelium samples. Immunohistochemical staining of VDR in squamous (B) and paired columnar epithelium (C) clearly shows a number of VDR positive nuclei in Barrett's tissue and its mere presence in the cytoplasm of epithelial cells. Arrows indicate some positively stained nuclei.

IDENTIFICATION OF A HAPLOTYPE-DEPENDENT GATA BINDING SITE IN THE VDR PROMOTER

The VDR gene encompasses at least 105 kb and contains a large 60 kb 5' region of non-coding exons, denominated as codon 1a through f (Suplementary Figure S1A). Genetic variation in VDR gene has been studied extensively in diseases of variety of organs including liver and intestine. In the esophagus, however, the role of genetic variation in VDR expression/function remains largely unknown. We investigated VDR gene polymorphisms possibly mediating intra-individual differences in the level of VDR transcription. The availability of multispecies genomic sequence allowed us to examine the sequence conservation across the transcriptional unit and indicated various highly pan-vertebrate conserved regions, especially near the non-coding exon 1c approximately 1,5 kb upstream of the translation start site (Figure 2). The strong evolutionary conservation in this region might be consistent with a role in transcriptional regulation. Among the fifteen tagging SNPs (Supplementary Figure S1B), two interesting polymorphisms were found in the exon 1c region, G1663C and C1453T. The latter converts the transcriptionally inert majority genotype into a canonical GATA binding site (supplementary Figure S2) and could thus be expected to alter VDR expression in GATA transcription factor expressing esophageal cell types, especially as abundant expression of GATA factors is a discerning characteristic of human proximal tract.



Abbreviated name for all mammalian species used:
Bos: *Bos taurus* (domestic cow)
Can: *Canis familiaris* (domestic dog)
Equ: *Equus caballus* (domestic horse)
Hom: *Homo sapiens* (human)
Mac: *Macaca mulatta* (rhesus macaque)
Mus: *Mus musculus* (house mouse)
Pan: *Pan troglodytes* (chimpanzee)
Rat: *Rattus norvegicus* (brown rat)

Figure 2. Comparative sequence analysis of multi-species alignment of the VDR 1c non-coding regulatory. The region containing the GT haplotype discussed in this manuscript is highly conserved in the human genome and 7 other mammalian species. The two SNP sites indicated are locus 1653G/C and 1453C/T and comprise haplotype assessed in this study.

To address the functional importance of the C1453T genotype, we tested the ability of VDR gene derived oligos of either the 1453C genotype or the 1453T genotype to bind GATA transcription factors in electrophoresis mobility shift assays. We have tested the binding of GATA1 transcription factor using an oligonucleotide bearing a SNP at locus 1453C/T together with nuclear extracts and antibodies against GATA1 (Figure 3A). Supershift experiments and competition experiments with GATA1-binding oligos indicated that GATA1 transcription factor binds to the oligonucleotide bearing the SNP 1453T mutation, whereas the oligonucleotide bearing SNP 1453C common allele does not show detectable binding (Figure 3B). Thus the 1453 polymorphism in the VDR results in a differential binding of GATA1 to VDR upstream sequences.

THE C1453T/G1663C HAPLOTYPES INFLUENCES ESOPHAGEAL VDR EXPRESSION

To address the functional consequences of the presence or absence of the GATA-binding site resulting from the C1453T polymorphism for VDR expression, esophageal biopsies were taken from the Barrett's segment from patients with BE and VDR expression levels were determined using qPCR for relation to the VDR C1453T and G1663C genotypes. The highest level of esophageal VDR mRNA were observed in BE patients caring the 1453C and 1663C (CC) haplotype (Figure 4). The expression of VDR was on average 4.5-fold higher in subjects caring two copies of the CC haplotype as compared to subjects carrying one or no CC haplotype alleles ($p=0.01$). Levels were lowest in tissue from patients carrying two alleles of the GT haplotype (Supplementary Table S1 and Figure S3), though this did not reached statistical significance. Thus the absence of the GATA binding sites in the CC haplotype results in higher esophageal VDR expression and thus presumably vitamin D sensitivity in this organ.

THE C1453T/G1663C HAPLOTYPE CONVEYING REDUCED VDR EXPRESSION IS ASSOCIATED WITH REDUCED RISK FOR NEOPLASM-ASSOCIATED ESOPHAGEAL DISEASE

A prediction from the observed association between VDR expression and malignant progression in the esophagus and the lower VDR expression in patients carrying the GT haplotype as compared the CC haplotype, would be that haplotype GT carriers are less at risk for developing (pre-)malignant association in the esophagus. To test this, genomic DNA was obtained from a group of 708 patients with reflux esophagitis, BE or EAC and compared to a group of 202 healthy controls without any previous symptoms of esophageal disease. Patient characteristics are given in Table 1. DNA samples were genotyped with the use of the high-throughput TaqMan allelic discrimination assays. As shown in Table 2, the VDR tagging SNPs of the exon 1c region, C1453T and G1663C, were found to be associated with BE and EAC. Thirteen other haplotype tagging SNPs across the VDR gene were found not significant with esophageal disease (data not shown).

A

WT: CCAGGGTGGTTGTCTA**C**CTGGATGTCACCTCTGACCTCTG

MUT: CCAGGGTGGTTGTCTA**T**CTGGATGTCACCTCTGACCTCTG

GATA

B

Mut VDR oligonucleotide	+	+	+	+	+	+	+
MEL nuclear extract	-	+	+	+	+	+	+
Self competition	-	-	+	-	-	-	-
Non-self competition	-	-	-	+	-	-	-
Complete non-self comp	-	-	-	-	+	-	-
GATA-1 competition	-	-	-	-	-	+	-
Anti GATA1	-	-	-	-	-	-	+

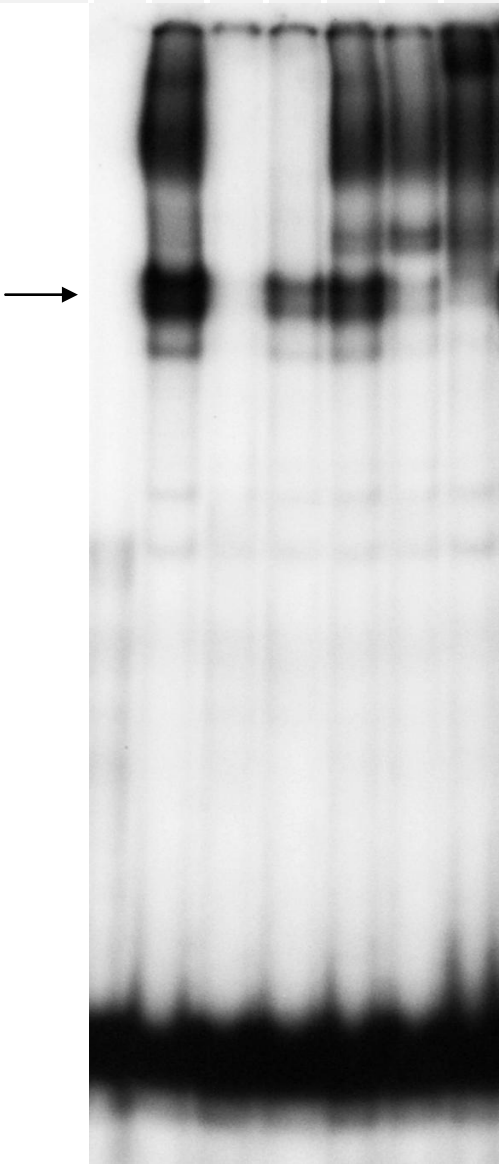


Figure 3. The VDR 1453T SNP represents a functional GATA1 binding motif. A) A putative GATA1 binding site is predicted at mutated 1453T SNP site. B) Gel shift assay using a radioactively labeled oligonucleotides from the C1453T region and nuclear extract of MEL cells. Arrow indicates the motility of the oligo-GATA1 complex. Lane 1, Mut oligo without nuclear extract; lane 2, Mut oligo with nuclear extract; lane 3, with 100× excess of unlabeled Mut oligo (self competitor); lane 4, with 100× excess of unlabeled WT oligo (Non-self competitor); lane 5, with complete non-self competitor; lane 6, with 100× excess unlabeled known GATA1 oligo; lane 7, 1 µg of anti-GATA1 monoclonal antibody. The signal found on the Mut oligo (lane 2) was almost completely eliminated by a 100-fold excess of unlabeled self competitor and a known GATA-1 oligonucleotide (lane 3 and 6) but not with WT and complete non-self oligonucleotides (lane 4 and 5). Labeled WT oligo did not result in GATA1 binding (data not shown).

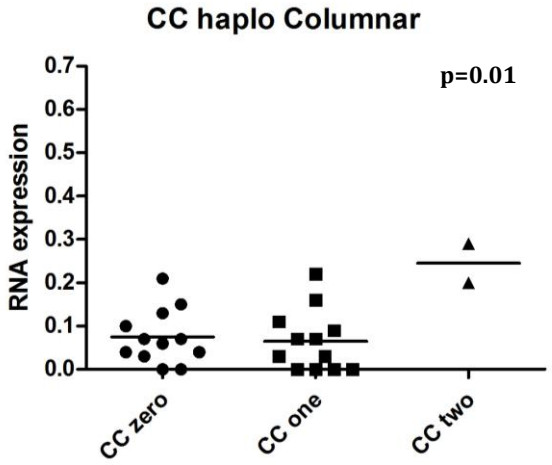


Figure 4. Lack of GATA1 binding site in the 1453C polymorphism promotes esophageal VDR expression. Barrett's biopsies from patients with BE were analyzed for VDR mRNA levels by qPCR. The esophageal expression of VDR was on average 4.5-fold higher in BE patients caring two copies of the 1453C and 1663C (CC) haplotype versus subjects caring one or no CC haplotype alleles (p=0.01). Levels were lowest in tissue from patients carrying two alleles of the GT haplotype (Supplementary Table S1).

As shown in Table 3, dose-risk allele analysis revealed that individuals carrying the 1633G/1453T haplotype were less susceptible to BE (OR 0.46; 95%CI 0.26-0.80), and EAC (OR 0.50; 95%CI 0.27-0.96). An independent BE replication cohort (n=150) was collected from the Academic Medical Center Amsterdam and closely mimicked the observations for the low VDR expressing genotype with respect to risk for contract the pre-metaplastic disease (OR 0.44; 95% CI 0.23-0.85; Table 3). Thus the reduced esophageal expression of the VDR associated with the GT haplotype (Table S1) correlates with reduced susceptibility for contracting premalignant disease as well as full-blown adenocarcinoma in the proximal tract.

Table 2. Genotype distribution of VDR SNPs G-1663C and C-1453T in healthy controls and patients

SNP locus	Genotype	Genotype, N (frequency)				
		HC	RE	BE	Repl BE	EAC
		N=202	N= 307	N=260	N=150	N=141
G1663C	G/G	127 (0.648)	159 (0.530)	157 (0.625)	72 (0.507)	76 (0.551)
	G/C	58 (0.296)	121 (0.403)	81 (0.323)	65 (0.458)	54 (0.391)
	C/C	11 (0.056)	20 (0.067)	13 (0.052)	5 (0.035)	8 (0.058)
	p-value	1	*0.03*	0.83	*0.01*	0.18
C1453T	C/C	53 (0.273)	107 (0.357)	85 (0.343)	49 (0.340)	49 (0.360)
	C/T	94 (0.485)	147 (0.490)	128 (0.516)	76 (0.528)	65 (0.478)
	T/T	47 (0.242)	46 (0.153)	35 (0.141)	19 (0.132)	22 (0.162)
	p-value	1	*0.02*	*0.02*	*0.03*	0.10

*Values in *italic* are significant at 95%CI. HC: healthy controls, RE: reflux esophagitis, BE: Barrett's esophagus, Repl BE: BE replication cohort, EAC: esophageal adenocarcinoma

Table 3. Dose-risk allele analysis of BE and rep BE compared to controls for c1453t /g1633c haplotypes

Risk allele	Total alleles (N)	Odds Ratio (95% CI)*			
		RE *versus* HC	BE *versus* HC	Repl BE *versus* HC	EAC *versus* HC
HAP1GT Zero	345	1	1	1	1
HAP1GT One	507	0.76 (0.50-1.15)	0.84 (0.54-1.30)	0.88 (0.54-1.43)	0.74 (0.45-1.22)
HAP1GT Two	169	*0.48 (0.28-0.81)*	*0.46 (0.26-0.80)*	*0.44 (0.23-0.85)*	*0.50 (0.27-0.96)*
HAP1GC Zero	442	1	1	1	1
HAP1GC One	444	1.37 (0.93-2.02)	*1.53 (1.02-2.30)*	*1.64 (1.03-2.61)*	1.08 (0.67-1.74)
HAP1GC Two	125	1.05 (0.57-1.93)	*1.84 (1.02-3.32)*	0.95 (0.44-2.05)	1.40 (0.71-2.77)
HAP1CC Zero	590	1	1	1	1
HAP1CC One	376	*1.65 (1.12-2.44)*	1.12 (0.74-1.68)	*1.98 (1.25-3.12)*	1.56 (0.98-2.48)
HAP1CC Two	57	1.24 (0.68-3.16)	0.96 (0.41-2.21)	0.80 (0.27-2.40)	1.22 (0.47-3.16)

*Values in *italic* are significant at 95%CI. HC: healthy controls, RE: reflux esophagitis, BE: Barrett's esophagus, Repl BE: BE replication cohort, EAC: esophageal adenocarcinoma.

DISCUSSION

The upward trend in the incidence of EAC remains on the rise and to date, surveillance strategies have not been able to attenuate, and is therefore hardly cost-effective. BE patients are left awaiting neoplasia or highly-morbid adenocarcinoma, at which point the fraction of patients benefiting from treatment are scant. Along with the recognized need for risk stratification in BE patients, this also calls for attention on identifying cohorts likely to respond to treatment on the mission for personalized chemoprevention.

According to epidemiological observations, vitamin D supplementation conveys chemopreventive properties in colorectal cancer and breast cancer, and its potential chemopreventive action in oncological disease attracts widespread attention. Nevertheless, clinical trials have been disappointing, and there is good agreement that identification of patient cohorts most likely to benefit from vitamin D supplementation would represent an important step forward. Studies on the genetic differences between different individuals influencing the tissue-specific responsiveness to vitamin D would represent rational avenue for detecting such cohorts. A major advantage of this type of genetic approach is that as DNA is identical in all tissues, so it can be easily obtained from blood. Thus, it provides an easy and relative cheap opportunity for early identification of population cohorts

that are at high risk of developing cancer or that are most likely to respond to chemopreventive therapy.

At present however, a major limitation of association studies exploring VDR polymorphisms in relation to complex-disease has been the small number of analyzed polymorphisms and thus, the lack of knowledge about the influence of and relation between other polymorphisms in the gene. In addition, the misconceptions about how such small biological effects could be translated into risk of disease have led to a number of controversies in the field. In the current study, we analyzed 15 tagging SNPs, previously determined by resequencing major relevant parts of the VDR gene,[31] to represent the common haplotypes for five haplotype blocks in potentially functional areas across the VDR gene in Caucasians. One of the polymorphisms studied, the C1453T mutation is associated with a substantially reduced risk for both the metaplastic cancer precursor lesion BE as well as full-blown adenocarcinoma and thus individuals carrying this mutation might be more likely to benefit from vitamin D supplementation.

Indeed, one of the major confounders hampering the chemopreventive action of vitamin D is the increased risk for adenocarcinoma of the esophagus. In apparent agreement, we observed that increased VDR expression and activation accompanies the progression from normal squamous epithelium to the columnar epithelial BE, supporting a negative role of VDR in this particular organ system. Interestingly, a haplotype of the C1453T and G1663C polymorphisms was found to be associated with reduced expression of the VDR in the esophagus and especially in Barrett's epithelium expecting to reduce vitamin D sensitivity here. A role of vitamin D in the progression from normal squamous epithelium to columnar BE epithelium is expected from the well known mutual positive interaction between VDR signaling and signaling of bone morphogenetic proteins; bone morphogenetic protein-4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells and is thus essential for precancerous process. The present study further supports this concept by establishing the upregulation of VDR signaling during the metaplastic process. Most importantly, however, by reducing vitamin D sensitivity in the esophagus, a rational explanation is provided as to how the C1453T polymorphism can reduce risk for esophageal cancer.

The mechanistic basis as to how the GT haplotype can reduce VDR expression seems to lie in the creation of a canonical GATA-binding site. Indeed, when tested in electrophoretic mobility shift assays, oligos containing 1453T polymorphism displayed strong GATA1 transcription factor binding, whereas oligos derived from the majority allele were not capable of doing so. GATA factors are well established mostly negative transcriptional regulators and accordingly the polymorphism reduces expression of the VDR. Accordingly, it was previously shown that IL-4 induces GATA1 which in subsequently represses VDR expression and enables

monocyte-derived dendritic cell differentiation within inflammatory sites.[32] This is a mechanism that could be happening in the esophagus during inflammation providing a rationale for the here-presented findings. Importantly, GATA transcription factors are highly expressed in the proximal tractus, where VDR expression has a negative influence of the cancerous process, as implied from theoretical (positive interaction with BMP signaling), epidemiological observations (dietary vitamin D intake and UV-B exposure are associated with increased risk for esophageal cancer) as wells as the observations made in the present study. In contrast, GATA transcription factor expression is much lower in the distal tractus, where the epidemiological evidence for chemopreventive effects of dietary vitamin D or UV-B exposure are the strongest. Thus we propose that individuals carrying GT haplotype are those most likely to benefit from vitamin D-based chemopreventive strategies. Confirmation of this notion obviously requires re-evaluation of previous trials involving dietary vitamin D supplementation, assessing the GT haplotype in the participants, but if confirmed the GT haplotype would represent the first polymorphism that stratifies individuals for the use of a practical chemopreventive strategy.

The importance of the GT haplotype in risk for BE, here reported to have an inverse relationship, is supported by the fact that it is more prevalent in African Americans (51.%) compared to Caucasians (40.2%), which could partly explain why BE is much more common in the West when compared with Africa.[33] The lower risk of BE in Asians though cannot be explained by the prevalence of the GT haplotype (32.3%).[31] (Supplementary Table S2)

In conclusion, this study serves as a proof of principle that single nucleotide genetic polymorphisms can modify the relationship between VDR expression and esophageal cancer risk, even at a premalignant stage. We believe in this perspective our data could represent an important advance in personalized chemopreventive medicine and may potentially change treatment recommendations for certain cohorts that are genetically, and/or otherwise, predisposed to EAC. The majority of Barrett's patients suffer from obesity, and adipose tissue is presumed to store vitamin D.[34] Development of an EAC often causes rapid weight loss due to dysphagia. In turn, this may lead to the fast liberation of excessive amounts of vitamin D from its adipose storage. This hypothesis underlines the relevance of our observations suggesting the adverse effects of vitamin D may be underestimated by introducing an extra safety concern for vitamin D supplementation in patients with BE or EAC. To investigate how serious the impact of vitamin D truly is, additional research is needed to confirm the aforementioned association and clarify the mechanisms by which vitamin D affects the risk of developing BE and its sequela EAC. At the same time, evidence from epidemiological studies mimicking true long-life effects of vitamin D are required to endorse the idea of personalized recommendations for vitamin D supplementation.[35]

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CHAPTER 6

BILE ACID-DEPENDENT, BUT CDX-2 INDEPENDENT, RECODING OF THE ENTERIC HOX CODE IN BARRETT'S ESOPHAGUS

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Manuscript in preparation

ABSTRACT

Background and aim: The mammalian *HOXA* cluster encodes master regulators of embryonic anterior to posterior specification, but their functioning in adult gastrointestinal physiology and disease remains poorly understood. Importantly, Barrett's Esophagus (BE) is a precancerous condition, induced by bile and acid reflux and defined by the presence of intestinal-type tissue in the esophagus. This positional misspecification in BE of posterior structures in an anterior organ raises questions as to the underlying mechanisms and especially the role of the *HOXA* cluster therein.

Methods: PCR is used to identify anterior-posterior patterning in murine and human gastrointestinal tract. Additionally, mRNA expression of all 11 members of the *HOXA* cluster, *CDX2* and *MUC2* is determined in esophageal biopsy samples of columnar epithelium and adjacent squamous epithelium from patients with BE (n=10) using Real-Time PCR. *HOXA11* protein expression is determined using immunohistochemistry. Cell culture experiments document effects of bile acids on *HOXA7, HOXA11, CDX2,* and *MUC2* expression.

Results: The gastrointestinal tract is characterised by predominance in expression of minor paralogue members of the *HOXA* cluster in anterior structures and major paralogue members in more posterior structures, much like the situation in the adult skeleton and nervous system[1,2,3] and in embryonic gastrulation. BE involves a reprogramming of this local enteric *HOXA* code to a posterior phenotype, marked by a notable repression of *HOXA7* and reactivation of *HOXA11*. In a human esophageal squamous cell line, bile acids repress *HOXA7* expression while provoking expression of posterior *HOXA11* and *MUC2* gene without concomitant effects on *CDX2*.

Conclusion: Our data and provide evidence for a role of the *HOXA* cluster in providing positional information in the gastrointestinal tract. We show reactivation of *HOXA* genes in the adult esophagus and suggest a direct *CDX2*-independent effect of bile acid on the reprogramming of the enteric *HOX* code is instrumental in the development of BE.

INTRODUCTION

The gastrointestinal tract is a remarkable body structure of different tissue and cell types, all carrying their own specific functions. The diversity of cells is the result of precise regulation of gene expression during development and cell differentiation of stem cells towards their mature phenotypic cell fate. Due to normal turnover or injury, specialised cells will often need to be replaced. To this end, master regulators of processes involved in cell and tissue differentiation need to be turned on and off, either in a programmed fashion or in response to the environment of the cell. If this regulation is disturbed this can potentially lead to tissue malformation or disease.

In the gastrointestinal tract, regulation and anterior to posterior patterning of specialised tissue is to a large extent dependent on the concerted action of two evolutionary highly conserved gene systems, the *Caudal-related homeobox* (*CDX*) transcription factor gene family and the genes from the *Homeobox* (*HOX*) cluster. *Caudal* was originally identified in *Drosophila* as a master regulator of embryonic antero-posterior patterning and has three mammalian homologues: *CDX1, CDX2* and *CDX4* in humans and *Cdx1, Cdx2* and *Cdx4* in mice. In *Drosophila*, *Caudal* specifies posterior body segments. *Cdx1*$^{-/-}$ mice exhibit anterior homeotic shifts in vertebral identity that involve the upper cervical vertebrae, whereas *Cdx2*$^{+/-}$ animals manifest similar homeotic defects more posteriorly, in the lower cervical and upper thoracic regions. *Cdx4*-null mice exhibit only a mild anterior transformation at a specific thoracic position with a very low penetration and no other abnormality. In mice, from 8.5 days onwards, *Cdx2* begins to be expressed in the posterior part of the gut and conditional ablation of *Cdx2* from early endoderm results in the replacement of the posterior intestinal columnar epithelium with squamous epithelium, a dramatic cell fate conversion caused by ectopic activation of the foregut/esophageal differentiation program. Conversely, ectopic expression of either *Cdx1* or *Cdx2* induces gastric intestinal metaplasia in transgenic mice.[4-6] Thus *Cdx* family members are without doubt important regulators of positional identity in the gut[7-8] and increased expression of *CDX2* in BE has been documented.[9] Nevertheless clear evidence that acquisition of *CDX2* is causative for BE is conspicuously lacking,[10] focussing attention on alternative mechanisms involved in the decoding of positional information.

HOX genes encode transcriptional regulatory proteins that control organogenesis and maintain tissue homeostasis and are key to developmental processes.[11] They all possess a sequence element of 180bp, the homeobox, which encodes a highly conserved 60-amino acid homeodomain, responsible for the recognition and binding of sequence-specific DNA motifs. Through binding to these regulatory regions, they control the expression of specific genes.[12] The *HOX* gene family[13] is known as the true homologues of the *Drosophila* homeotic genes controlling the body plan of this insect.[1] In mammals, there are at least 39 *HOX* genes clustered into 13 paralogue

groups distributed on chromosomes 7 (*HOXA*), 17 (*HOXB*), 12 (*HOXC*), and 2 (*HOXD*).[13-15] HOX genes are well known for their function in conferring positional information along the anteroposterior body axis during embryonic morphogenesis exhibiting the colinearity property; expressing from 3′-anterior to 5′-posterior.[1] Though their biological function in embryos is the best studied, *HOX* genes also express in some normal adult organs suggesting a role in the maintenance of tissue specificity.[16] Additionally, expression patterns have been studied in various human malignancies, including cancers of breast,[17] colon,[18-19] esophagus,[20] lung,[21-22] kidney,[23] uterus,[24] and prostate.[25-26] Some studies report altered expression in normal and cancerous tissue, either loss or gain of expression,[27] and a growing body of evidence suggests that *HOX* genes are involved in oncogenesis and malignant progression. The fundamental importance of *HOX* gene in anterior-posterior identity, warrants further research into *HOX* gene action in the gut.

Apart from a lack of fundamental knowledge as to the nature of the *HOX* signals present in the alimentary tract, also the genetic integration between *Cdx* gene family-derived and *Hox* gene family-derived signals in the gastrointestinal system remains unclear. Generally in vertebrate axis formation, there is a strong reciprocal interaction between *Hox* signals and *Cdx2* signals, *e.g.* genetic disruption of the murine homeobox gene *Cdx1* affects axial skeletal identities by altering the mesodermal expression domains of Hox genes.[7] In animals with an endodermal-specific *Cdx2* deletion, array data suggested that the homeotic transformation from columnar epithelium does not involve changes in the enteric Hox code. Whether Cdx2 signals and Hox signals act independently elsewhere in the gastrointestinal tract remains unclear.

A typical example of specialised differentiation in the gastrointestinal tract is Barrett's esophagus (BE). BE is a metaplasia of the esophageal epithelium defined as replacement of the normal multi-layered squamous epithelium by crypt-structured intestinal epithelium with presence of goblet cells. BE develops as a complication of chronic gastro-esophageal reflux disease in a subset (5-12%) of reflux patients, and is the only known precursor of esophageal adenocarcinoma (EAC).[28-29] While insight into the genetic and epigenetic events associated with progression of BE to EAC is growing, preventing or reversing BE currently appears the best option for avoiding development of EAC. The past decades, therapeutic strategies have been directed at prevention and reversal of chemical injury to the esophageal mucosa caused by regurgitating bile and acid, but development of rational therapy is hampered by a lack of understanding as to the mechanisms that underlie the homeotic change from multilayered squamous epithelium to a single layer of columnar cells. As BE appears to involve the acquisition by anterior gut structures of a more posterior phenotype, misinterpretation of positional information is likely to be involved in the pathogenesis of this disease. Thus, more than sufficient evidence to support the rationale that key morphogenic regulators, such as *HOX* and *CDX2* genes are involved in BE. As aforementioned reports indicate, *CDX2* has been extensively

studied. However, the regulation of *HOX* genes and their relation with *CDX2* in BE has not been investigated. *HOXA* cluster genes in particular, were shown to be expressed in proliferating epithelial cells of the adult intestine and in colon adenocarcinoma. We therefore chose to investigate the expression of all paralogues of the *HOXA* gene cluster in BE. We observe that BE is characterised by more posterior *HOX* coding as compared to squamous epithelium. Furthermore, *in vitro,* bile acids were capable of transforming esophageal cells from anterior *HOX* coding to a posterior *HOX* expression pattern without a concomitant effect on *CDX2*. We propose that bile acids contribute to pathogenesis of BE directly through reprogramming of the enteric *HOX* code.

MATERIALS AND METHODS

PATIENT AND MOUSE SAMPLES

Biopsy specimens from 10 BE patients were selected. Patients were assessed at the endoscopy unit of the Erasmus Medical Centre Rotterdam and all patients were endoscopically diagnosed with BE with histologically confirmed specialised intestinal metaplasia without high-grade dysplasia. From each patient, duplicate biopsies of both the squamous and the columnar epithelium were collected approximately 2 cm above and 2 cm below the squamocolumnar junction. From transgenic knock-in mice, carrying one extra copy of all of the *HoxA* cluster genes, the entire gut was resected and dissected into duodenum and jejunum, ileum and colon. Next, epithelial layer was stripped from the muscularis mucosa and both layers were homogenised and analysed separately.

REAL-TIME PCR MRNA QUANTIFICATION

Total RNA was extracted from tissue biopsies using TriReagent (Sigma, St Louis, MO) and purified using an RNeasy micro column kit (Qiagen, Hilden, CA). One-fortieth of a 1 µg cDNA synthesis reaction (iScript cDNA Synthesis Kit; Bio-Rad) was used in a 25 µl Real Time-PCR using SYBR GreenER (Invitrogen, Carlsbad, CA). Mouse and human specific primers used are given in supplementary Table 1. Duplicate samples were run three times in independent PCR-runs and the average levels of the genes of interest were normalized to GAPDH using the ΔCt method.[30]

CELL CULTURE

The human squamous epithelial cell line HET1A was obtained from the ATCC. HET1A cells were cultured in flasks (Greiner Bio-One) treated with FNC coating mix containing serum-free BRFF-EPM2 medium (10P's Axxora) supplemented with 100 units/ml penicilline and streptomycin. Cells were maintained routinely at 37°C in

5% CO_2 humidified atmosphere. After a period of at least 24 hrs to allow cells to adhere they were stimulated with 10 μM deoxycholic acid for 18 days, with acidic medium at pH 4, or with a bile acid cocktail containing GCA, TCA, GDCA, GCDCA and DCA in a total concentration of 50 or 100 μM for 24 hrs. During harvesting, cells were rinsed with 0.05% (w/v) trypsin-EDTA supplemented with 0.5% polyvinylpyrrolidone (Sigma-Aldrich) before trypsinisation with 0.25% (w/v) trypsin-EDTA. Culture medium with soy-bean trypsin inhibitor (Invitrogen) was added to stop trypsinisation.

IMMUNOHISTOCHEMICAL STAININGS

From formalin-fixed, paraffin-embedded tissue blocks, sequential sections were taken and mounted on adhesive slides (Starfrost, Berlin, Germany). After deparaffinization in xylene and dehydration in alcohol, endogenous peroxidase was inactivated by incubation with 1% hydrogen peroxidase in methanol for 20 min. Microwave pretreatment in TRIS/EDTA pH 9.0 was applied for 10 min to retrieve antigens. Sections were treated with 10% normal human plasma and 10% goat serum to block non-specific staining. Incubation with mouse anti-human HOXA11 antibody (1:200; Abnova) for 1 hour took place at RT, followed by polyclonal goat anti-mouse (1:200; Dako, Denmark). After 45 min of incubation with streptavidin–horseradish peroxidase (HRP) (Dako) HOXA11 was visualized using diaminobenzidine as a substrate and counterstained with hematoxillin. As a negative control the first antibody was omitted and an isotype control was included. Tissue of transgenic mouse colon was taken as a positive control. Sections were evaluated using light microscopy (Axioskop 20; Zeiss). Pictures were taken and analysed using Nikon software (NisElements 2008).

RESULTS

AN EPITHELIUM-SPECIFIC ENTERIC *HOX* CODE DEFINES ANTERIOR TO POSTERIOR POSITIONAL IDENTITY

The *HOXA* cluster plays a central role in establishing the initial body plan by providing positional information along the anterior-posterior body and limb axis, with the minor paralogue *HOX* genes exhibiting a more anteriorally restricted expression pattern as compared to major paralogue *HOX* genes. For instance, in the nervous system *HOXA1* is only expressed in the rostal telencephalon, whereas *HOXA13* expression extends through the entire spinal cord. Whether the *HOXA* cluster intergrates positional information in the gut in a similar manner, however, has not been investigated. Hence we investigated mRNA expression of *HOX* genes in the different epithelial regions of the mammalian gut. To this end RNA isolated from different parts of the murine gastrointestinal system (see material and methods) and the anterior aspect of expression of different member of the *HOXA* cluster was

determined. In agreement with the information obtained from other organ systems expression of different members of *HOXA* cluster in the murine enteric epithelium is strongly positionally dependent, with minor paralogue members of the *HOXA* cluster being expressed at more anterior regions and major members showing a more pronounced posterior expression pattern (Figure 1A). In contrast when the muscularis mucosa was investigated in the same fashion, apparent position-independent expression of *HOXA* cluster genes was detected, likely necessary to maintain musculature structure integrity (Figure 1B). To investigate whether the same situation holds true for humans, we also compared relative expression of the different members of the *HOXA* cluster in biopsies of human esophagus and colon and again we observe that the minor paralogue members of the *HOXA* cluster are strongly expressed in the esophagus, whereas the human colon is characterised by strong expression of the major paralogue members of this cluster (Figure 1C), with the exception of *HOXA2* which stably expresses in anterior as well as posterior structures of human and mouse. Thus, RNA data suggests the existence of an enteric *HOX* code providing positional information in the gut.

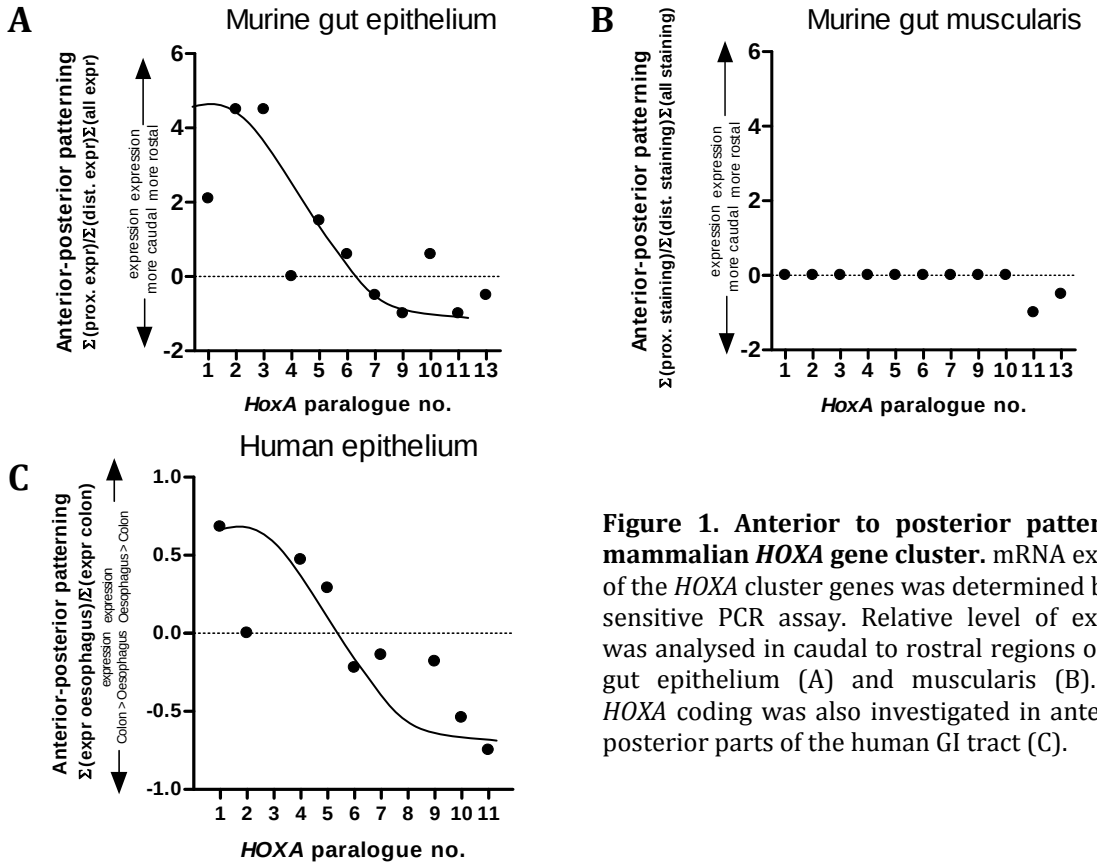

Figure 1. Anterior to posterior patterning of mammalian *HOXA* gene cluster. mRNA expression of the *HOXA* cluster genes was determined by highly sensitive PCR assay. Relative level of expression was analysed in caudal to rostral regions of murine gut epithelium (A) and muscularis (B). Enteric *HOXA* coding was also investigated in anterior and posterior parts of the human GI tract (C).

IN BARRETT'S ESOPHAGUS THE ENTERIC *HOX* IS REPROGRAMMED TO MORE CAUDAL PHENOTYPES

Having established an enteric *HOX* code in which minor *HOX* genes are associated with more anterior positional identity and major *HOX* genes with a more posterior location, we wondered whether the apparent caudal identity of the epithelium in Barrett's esophagus is associated with altered *HOX* coding. Thus biopsies were taken from both the Barrett's segment of 10 patients as well as the adjacent normal squamous epithelium and investigated for expression of the genes of *HOXA* cluster using real-time PCR. As a control also expression of the intestinal mucin *MUC2* and the esophagus-specific cytokeratin *CTK14* were investigated as well and showed that in our hands in BE *MUC2* is strongly upregulated, whereas *CTK14* is downregulated, confirming the validity of the assay system and the integrity of the tissue (Supplementary Figure 1). Importantly, expression of minor paralogue *HOXA* genes, which are associated with more anterior intestinal phenotypes is upregulated in squamous epithelium as compared to Barrett's esophagus. Conversely, expression of major paralogue *HOXA* genes, which are associated with posterior intestinal identity, is upregulated in the Barrett's segment (Figure 2A). *HOXA11* was chosen for quantification in 7 patients by Real-Time PCR because RT-PCR data showed a remarkable difference of mRNA levels in BE compared to squamous epithelium. Indeed, *HOXA11* expressed on average 2.8–fold higher compared to matching squamous epithelium (Figure 2B). Next, we sought to confirm our data on the protein level using immunohistochemistry. Heterologous expression of *HOX* genes in Cos7 cells enabled us to identify an antiserum that specifically and reliably stains for *HOXA11* in immunohistochemical experimentation (Supplementary Figure 2) and we employed this serum to stain biopsies taken from human BE tissue and adjacent squamous tissue (Figure 2C-E). HOXA11 staining is very weak to undetectable in the squamous epithelium of the esophagus (Figure 2D), where HOXA11 nuclear staining is evident in BE tissue (Figure 2E). These observations demonstrate that BE involves the reactivation of HOXA11.

BILE ACID REPROGRAMS THE ENTERIC *HOX* CODE TO A POSTERIOR PHENOTYPE

It is well established that the development of BE is mediated by the reflux and subsequent exposure of the squamous epithelium to acid and bile, but the underlying molecular mechanisms remain largely unresolved. To address the possible functional relevance of the enteric *HOXA* code reprogramming in the mediating the effects of reflux in the development of BE, we resorted to an in vitro system in which cultures of the HET-1A cell line derived from squamous esophageal epithelium were exposed to a cocktail of various bile acids. Indeed, when cells we exposed to bile acids, the intestinal mucin *MUC2* was induced in a concentration dependent fashion, showing that important aspects of BE can be mimicked in this *in vitro* system (Figure 3A). Importantly, this bile acid-dependent induction of *MUC2* was close mirrored by *HOXA11* expression (Figure 3B). Hence, the bile acid-

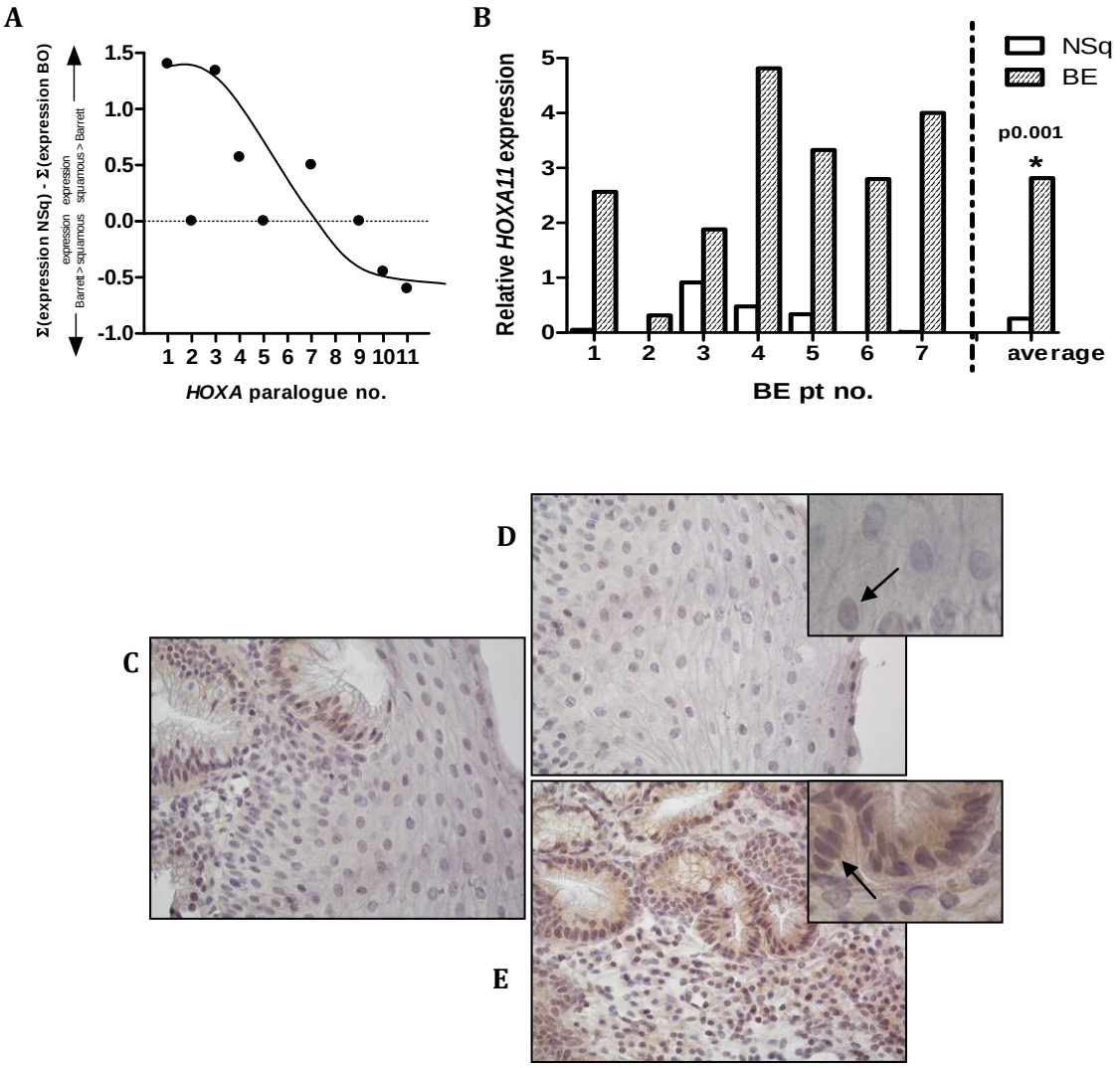

Figure 2. Aberrant HOXA expression in Barrett's compared to adjacent squamous epithelium. Minor paralogues of the *HOXA* cluster are downregulated or turned off in Barrett's tissue, whereas major paralogue *HOXA* genes appear to be upregulated or switched on in Barrett's compared to adjacent squamous epithelium. (A) Highly upregulated *HOXA11* was quantified in 7 patients using Real-Time PCR (B), reporting an average 2.8-fold induction (p=0.0011). Activation and functional expression was explored at a protein level using immunohistochemical staining on Barrett's and directly adjacent squamous tissue (C). Nuclear HOXA11 is clearly detected in Barrett's tissue (D), but is not, or to a much lesser extent, present in adjacent squamous epithelium (E). Magnification 400x with inlays of 1000x magnification.

dependent acquisition of the BE phenotype is accompanied by acquisition of expression of posterior *HOXA11* gene. Conversely, the anterior *HOXA7* gene is downregulated by bile acid treatment (Figure 3C). Thus, bile acid treatment of human esophageal squamous cell line suggests that in BE the *HOXA* code is reprogrammed towards a posterior phenotype by suppression of anterior and upregulation of posterior *HOXA* genes.

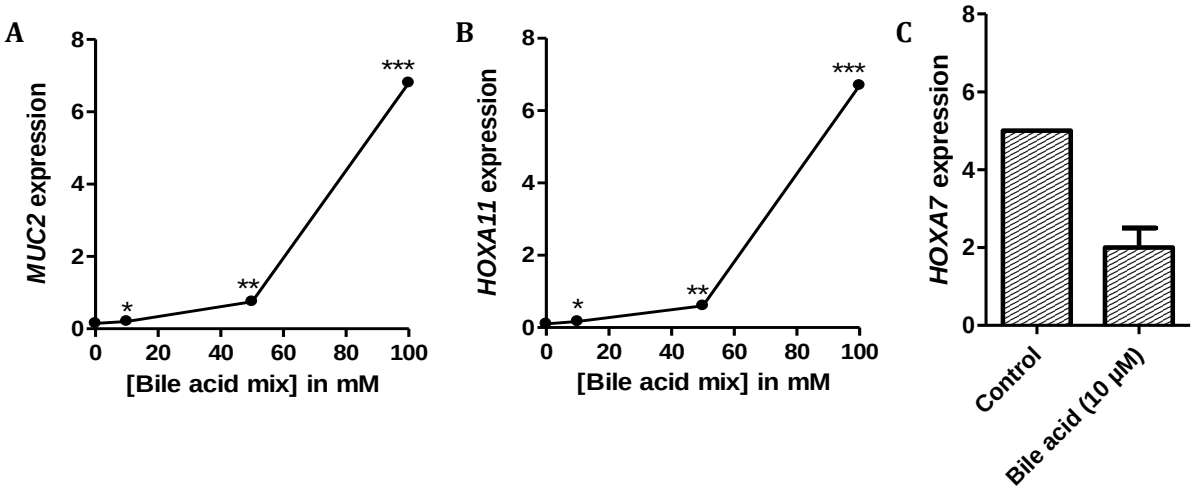

Figure 3. Bile acid-dependent regulation of *HOXA* genes and intestinal *MUC2* in a human esophageal squamous cell line. A bile acid mixture resembling human refluxate (see materials and methods) commonly used in our lab induced *HOXA11* (A) and *MUC2* (B) in HET-1A cells in a dose-dependent manner. Controversely, *HOXA7* was significantly suppressed in HET-1A cells upon stimulation with as little as 10 μM of bile acid (C).

ACQUISITION OF POSTERIOR PHENOTYPES IS INDEPENDENT OF CDX2

CDX2 represents an important posteriorizing signal in the body and is necessary and sufficient for intestinal metaplasia in the stomach. In most metazoan organ systems it induces caudal *HOX* coding and antagonises anterior *HOX* signals. In a mouse containing an endoderm-specific deletion of *CDX2*, however, squamous epithelium in the colon was observed without concomitant -as judged DNA array analysis- changes in expression of *HOX* genes, raising questions as to the importance of *CDX2* in BE in general and especially its relation to *HOX* coding. In line with the aforementioned observation, we observe strong induction of *CDX2* in BE (Supplementary Figure 2), however when *CDX2* expression and *MUC2* expression in the same human BE samples are compared, only a moderate correlation (R^2=0.43) is observed, whereas *HOXA11* and *MUC2* expression exhibit strong correlation (R^2=0.82). This suggests that in BE the expression of posterior *HOXA* genes is more important for maintaining the caudal phenotype as compared to *CDX2*. Indeed, induction of *MUC2* expression by bile acids in HET-1A cells is not accompanied by an induction of *CDX2*, expression of this gene being undetectable before or after bile acid application even in RT-PCR assays (data not shown). Thus, induction of posterior *HOX* genes seems more important for establishing the BE phenotype as induction of *CDX2*.

DISCUSSION

The formation of intestinal metaplasia in the esophagus suggests that BE involves a deregulation in the genetic decoding of positional information in the intestinal tract. As the *HOXA* cluster is a cardinal component in the molecular mechanisms that mediate proper gene expression relative to the anterior-posterior axis of the body, we were prompted to investigate the importance of *HOXA* in the pathogenesis of BE. Indeed we observe that in the mammalian gut epithelium *HOXA* genes display position-specific expression, forming an enteric *HOX* code, anterior structures being defined by relative overexpression of genes of the minor paralogue members of the *HOXA* cluster, whereas further down the tractus expression of *HOXA* genes of the major paralogues become dominant. Furthermore, we observe that in BE a reprogramming of the *HOX* coding to a more posterior phenotype is evident, expression of posterior *HOXA* genes correlating well with the expression of the intestinal marker *MUC2*. Finally, we observe that bile acids are capable of regulating *HOXA* coding in an esophageal cell line, and that concomitantly *MUC2* is induced. We conclude that bile acid-mediated reprogramming of *HOX* coding can contribute to the pathogenesis of BE.

Like the *HOXA* cluster, *CDX2* is an important source of positional information for gene expression. For intestinal metaplasia in the stomach, a role for *CDX2* is well established, but for BE the picture is less clear. Ectopic expression of *CDX2* in the esophagus has a very mild phenotype, cells acquiring some characteristics of columnar cells but cells obtain at best a very intermediate phenotype, retaining most characteristics of squamous epithelium. Like others, we observe high expression of *CDX2* in BE, but importantly, correlation between this expression and *MUC2* induction is only moderate. Furthermore, we observe in HET-1A cells that low concentrations of bile acids are capable of inducing *MUC2* and reprogramming of the *HOXA* coding without a concomitant induction of *CDX2*. Thus effects of *HOX* reprogramming on cellular phenotype in the esophagus does not require *CDX2*. In a rodent squamous cell line of bile acid produce some induction of the *CDX2* promoter in luciferase assays,[31-32] whereas bile acids demethylate the *CDX2* promoter in HET-1A cells and in combination with acid produce also strong *CDX2* expression.[33] Thus it is possible that full transformation of squamous epithelium to BE requires first a *HOX*-dependent demethylation of the *CDX2* promoter followed by acid-dependent expression of this gene. Alternatively, in the colon, mice that have an endodermal specific deletion of *CDX2* obtain a squamous phenotype without reprogramming of enteric *HOX* coding, thus it possible that the *HOX* positional system and *CDX2*–dependent axial signaling are in the colon operating more independent as elsewhere, *CDX2* mainly serving to repress the squamous phenotype, whereas *HOX* signaling is more important in determining positional identity. Further

experimentation is thus needed to exactly clarify the relation between *HOX* signaling and *CDX2* signaling.

The discovery that *HOX* coding is altered in BE has important consequences for thinking of BE as expression of intestinal markers in this disease is sometimes interpreted as a misexpression of a subset of intestinal genes following bile and acid-dependent insult and genomic instability with oncogenenic transformation being the result of further escalation of genome dysregulation involved, while others view BE as a true metaplasia with the oncological transformation resulting from the ectopic localisation of the intestinal tissue involved. Our results that BE coincides with reprogramming of the *HOX* positional machinery lend further credit to the second school of thought, although it should be kept in mind that *HOX* genes are only part of the positional machinery and other systems (like *CDX2*) must be involved as well.

In conclusion, we find the enteric *HOX* coding is present and functional in the adult gastrointestinal tract. Our data suggests *HOX* reprogramming controls important aspects of intestinal identity as observed in BE where esophageal epithelium transforms into a columnar phenotype. This may be directly triggered by bile acid induced expression of *HOX* genes. *HOX* genes of the 7 and 11 paralogue are herein suggested to be important players. In our hands, this function appeared to be largely independent of *CDX2,* generally presumed as an early BE marker.

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CHAPTER 7

COMMON VARIANTS AT THE MHC LOCUS AND AT CHROMOSOME 16Q24.1 PREDISPOSE TO BARRETT'S ESOPHAGUS

The Esophageal Adenocarcinoma Genetics Consortium and the Wellcome Trust Case Control Consortium 2*

*A list of authors and their affiliations appears at the end of the paper; membership of both consortia is listed in Supplementary Information.

Submitted

ABSTRACT

Background and aim: Barrett's Esophagus (BE) is an increasingly common disease that is strongly associated with reflux of stomach acid and usually a hiatus hernia. BE develops on a background of chronic esophagitis and strongly predisposes to esophageal adenocarcinoma (EAC), a tumor with a very poor prognosis.

Methods: We have undertaken the first genome-wide association study on BE, comprising 1,853 BE cases and 5,175 controls in the discovery and 1,586 cases and 7,966 controls in the replication, all from northern Europe.

Results: Two regions were associated with BE risk: chromosome 6p21, rs9257809 ($P_{combined}=4.30x10^{-9}$) and chromosome 16q24 rs9936833 ($P_{combined}=2.68x10^{-9}$). The top SNP on chromosome 6p21 is within the major histocompatibility complex, and the closest protein-coding gene to rs9936833 on chromosome 16q24 is *FOXF1,* which is implicated in esophageal development. We found evidence that the risk alleles for SNPs predisposing to obesity also increase BE risk, and further analysis shows the genetic component of BE is mediated by many common variants of small effect.

INTRODUCTION

Barrett's Esophagus (BE) is the second most common pre-malignant lesion in the Western world after large bowel polyps. BE affects over 2% of the adult population and, unlike bowel polyps, lacks any proven effective therapy [1]. In the majority of cases, BE is associated with chronic gastro-esophageal reflux disease (GERD), including esophagitis [2,3]. In addition there are structural changes, mainly hiatus hernia, in the lower esophagus in over 80% of BE patients [4]. This allows both acid and bile to remain immediately adjacent to the esophageal epithelium. The annual risk of esophageal adenocarcinoma (EAC) in BE is approximately 0.5-1% per year [5,6]. Notably the incidence of EAC has been rising by 3% each year for the last 30 years; it is now the fifth commonest cancer in the UK. Despite modern multimodality therapy, the prognosis of EAC remains poor, with a 9-15% 5-year survival [7,8].

The etiology of BE is not well characterised. Environmental factors, such as diet, are weakly associated with GERD, BE and EAC, and obesity is a known risk factor for all three conditions [9]. There is also evidence of causal genetic factors; the relative risks are increased 2-4 fold for GERD, BE and EAC when one first-degree relative is affected [10-15]. However extensive candidate gene and linkage searches have, to date, failed to identify genetic variants that are associated with BE risk (unpublished findings from the international panel; BArrett's Dysplasia and CArcinoma Task force (BAD CAT) 2011 Chair Jankowski, J)

As part of the Wellcome Trust Case Control Consortium 2 (WTCCC2) study of 15 common disorders and traits, we present the results of the first genome-wide association study on BE susceptibility. Using cohorts from UK and Dutch populations we identify two variants associated with BE, and find evidence for hundreds of common variants with small effects which contribute to BE risk.

METHODS

SAMPLES

Discovery. We ascertained cases of histologically-confirmed BE through the United Kingdom-based ASPECT clinical trial of proton pump-inhibitor (esomeprazole) and aspirin as preventive agents for progression of BE to EAC, the Chemoprevention of Premalignant Intestinal Neoplasia (ChOPIN) genetic trial and the Esophageal Adenocarcinoma GenE (EAGLE) consortia. We used strict and objective criteria for diagnosis of Barrett's oesophagus (see Supplementary). The case samples were collected at centers UK-wide (Supplementary Table 1). All case subjects met the accepted international criteria for the endoscopic and histological classification of BE.

Controls were taken from the Wellcome Trust Case Control Consortium 2 (WTCCC2) set, made up of samples from the 1958 British Birth Cohort (58C) and the National Blood Service collection (UKBS).

Samples were genotyped at the Wellcome Trust Sanger Institute (WTSI); cases on the Illumina Human660W-Quad array, and controls on the Illumina Human1.2M-Duo array. The primary analysis was performed on the overlapping set of SNPs. Prior to any exclusions, the full data set comprised of 1,991 cases and 5,667 controls.

UK Replication. Following quality control the UK replication totaled 1,108 cases and 6,819 controls. The controls were from the PoBI cohort (2,578) [17] and 58C (4,241) samples that were not genotyped in the discovery phase. The case and 58C control samples were genotyped on the Illumina Immunochip and the PoBI samples were genotyped on the Illumina 1.2M-Duo array.

Dutch Replication. The Dutch replication cohort consisted of 478 cases and 1,147 controls. These samples were all genotyped on the Illumina Immunochip but in two different locations; the case samples were genotyped at WTSI and the control samples were genotyped as described in [36].

Replication samples were used as part of a shallow replication cohort, using data for ~100 SNPs in the UK and <20SNPs in the Dutch sample. The UK cohort was also used for a larger *en masse* analysis using the summary statistics for ~2,000 SNPs.

DNA SAMPLE PREPARATION

Genomic DNA for all discovery and UK replication samples was shipped to the Wellcome Trust Sanger Institute (WTSI), Cambridge. DNA concentrations were quantified using a PicoGreen assay (Invitrogen) and an aliquot assayed by agarose gel electrophoresis. In order to track sample identity, ~30 SNPs, including sex chromosome markers, were typed on the Sequenom platform prior to entry to the whole genome genotyping pipeline. See Supplementary for further details.

QUALITY CONTROL

Samples. Sample exclusions were based on four genome-wide summary statistics of the genotyping data designed to be sensitive to possible sources of heterogeneity: fraction of missing genotypes, autosomal heterozygosity, a measure of African and Asian ancestry (defined by a principal component analysis of the HapMap2 data) and the average difference in the probe intensities across SNPs. By modeling the distribution of each of these summaries as a mixture, we inferred outlying individuals and excluded them from analysis. Furthermore, we excluded one of each pair of individuals showing greater than 5% identity by descent by inferring chromosomal sharing at a genome-wide subset of SNPs. To reduce the risk of errors through sample swaps, we also removed samples for which the reported and

genetically-determined gender were discordant, or where Illumina array based genotypes disagreed with more than 10% of the Sequenom genotypes which were typed as part of sample preparation described above. After sample quality control, 1,853 cases and 5,175 control samples remained for analysis (Supplementary Table 10).

SNPs. For all arrays, normalised probe intensities were exported using the BeadStudio program and genotypes were called using Illuminus. For the purposes of quality control, SNPs were excluded from analysis if any of the data sets (58C, UKBS or BE) had a minor allele frequency less than 0.01%, a significant departure from Hardy Weinberg Equilibrium ($P<10^{-20}$) or a significant association with the plate on which the samples were assayed ($P<10^{-6}$). SNPs were also excluded if the observed statistical (Fisher) information about the allele frequency was less than 98% of the information contained in a hypothetical sample of the same size and expected MAF but with no missing data. Additional SNPs were removed following visual inspection of cluster plots. In total 521,744 SNPs were available for analysis after quality control.

HLA IMPUTATION

HLA*IMP [21] was used to impute classical HLA alleles using SNP genotype data for the discovery phase samples. The "MS_NBS" set of SNPs was selected to inform the imputation. This SNP set had been selected by the HLA*IMP team for use in WTCCC2 studies. Best guess alleles imputed with a probability score greater than 0.7 were analyzed. 145 different alleles imputed in all three datasets were investigated in the discovery, and alleles with the most significant associations were then imputed in the replication data. The "IMMUNOCHIP" set of SNPs, selected by the HLA*IMP team was used to inform the replication phase HLA imputation.

CIRCUMFERENTIAL AND MAXIMAL EXTENT PHENOTYPES

Information on the length of the Barrett's segment was available for a subset of discovery and replication phase samples. Where baseline measurements were not available, the earliest measurement taken after baseline was used. If the C value was larger than the M value (clinically not possible) or if either value exceeded 25cm, an individual was excluded from the analysis. Of the discovery phase individuals, 1,744 had C measurements and 1,618 had M measurements (after quality control), C mean=4.05 (range 0-22); M mean=4.60 (range 1-24). M measurements were available for 1,015 of the UK replication participants and for 240 of the Dutch replication participants. UK M mean=4.66 (range 1-23), Dutch M mean=4.44 (range 1-15). Neither C nor M phenotypes are normally distributed so a square root transformation was necessary.

STATISTICAL ANALYSIS

Genome-wide case-control analysis was performed using frequentist tests, under a missing data logistic regression model, as implemented in the program SNPTEST (http://www.stats.ox.ac.uk/~marchini/software/gwas/snptest.html). Unless otherwise stated, we assumed a multiplicative model for allelic risk by encoding the genotypes at each SNP as a discrete explanatory variable with an indicator of case status as the binary response. Quantitative C and M measurements were analysed using frequentist tests under a missing data linear regression model, as implemented in SNPTEST.

EN MASSE ANALYSIS

This analysis was carried out on the UK data only, and in order to reduce possible population structure that such analyses are sensitive to, we restricted the replication control set to the UKBS and 58C individuals.

SNPs with MAF > 0.01 which were genotyped in both the discovery (Illumina 670K and Ilumina 1.2M-Duo) and the replication (Illumina Immunochip) were pruned to remove strong linkage disequilibrium. This is done by ranking the SNPs by Bayes factor and successively selecting SNPs from the top so that they are at least 0.125cM plus 25kb away from any SNP that have already been selected. We obtained 7,673 SNPs from a total of 28,972 (after quality control) that were typed in all data sets.

The Sign Test compares the direction of effect for each SNP in the discovery and replication samples. Under the null, the direction of effect in the discovery and replication are independent.

The disease-score test aims to measure indirectly the collective effect of many weakly associated alleles that tend to show only very small allele frequency differences between cases and controls, but nonetheless have higher average association test statistics and lower P values than null loci. We determined the risk allele and odds ratio for each pruned SNP from the discovery data. Then, we used this information in the replication data to calculate the “score” for each individual, which is the number of risk alleles carried by each individual weighted by the log of the odds ratio from the discovery data. We tested a logistic regression model of disease status on the score, conditioning on the first principal component, to control for population structure, and the number of missing genotypes (called with maximum probability < 0.9), to control for potential differences in genotyping rate, as covariates. Under the null, the risk alleles and odds ratios in the discovery and replication samples are independent.

RESULTS

For the discovery analysis cases with histologically confirmed BE (see methods) were recruited from 86 sites across the UK (Supplementary Table 1). Population controls were taken from the WTCCC2 common set of 1958 Birth Cohort (58C) and National Blood Service (UKBS) samples. The case individuals were genotyped on the Illumina 660W-Quad and controls were genotyped on the Illumina custom Human 1.2M-Duo arrays, with the analysis performed on the overlapping set of SNPs. Following quality control (see Methods), a total of 521,744 SNPs typed in 1,853 BE cases and 5,175 controls (2,501 UKBS and 2,674 58C) were taken into the discovery analysis.

Association analysis was carried out under a logistic regression model as implemented in SNPTEST (http://www.stats.ox.ac.uk/~marchini/software/gwas/snptest.html). The genomic over-dispersion factor [16] λ was 1.10 and this was reduced to 1.05 when incorporating the first two principal components (PC) as covariates. For all the following results presented, unless otherwise stated, the first two PC were used as covariates.

Following analysis of the genome wide association results (Figure 1), a total of 100 SNPs were chosen for replication, all of which showed evidence of association in the discovery data ($P<5x10^{-4}$) (see Supplementary Table 2).

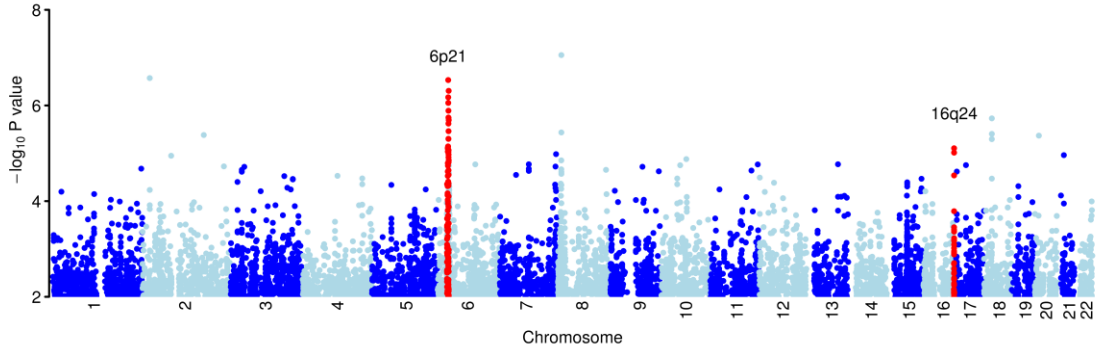

Figure 1. Plot of the genome-wide association results after fitting the multiplicative model in SNPTEST. Results shown for the 521,744 SNPs passing quality control filters. Chromosomes are colored dark blue and light blue alternatively, as labeled on the x-axis. The y-axis shows the –log10 P values. Regions in red show the loci newly identified as associated with BE, as described in Table 1.

Replication was attempted in two cohorts, one collected in the UK and the other in the Netherlands. The UK collection comprised of 1,108 cases from 77 sites (Supplementary Table 3), and 6,819 controls from the 58C control dataset, and the People of the British Isles (PoBI) collection [17]. The Dutch collection contained 478 cases from 4 sites (Supplementary Table 4) and 1,147 controls. The Dutch

replication dataset, the UK cases and the UKBS and 58C controls were genotyped on the Illumina Immunochip, a custom designed chip containing 2,400 SNPs selected from this study. The PoBI cohort was genotyped on the custom Illumina Human custom 1.2M-Duo array. Meta-analysis of the discovery data and a preliminary release of the UK replication data identified 16 SNPs with promising evidence of disease association ($P_{combined} < 10^{-5}$); these were taken through to replication in the Dutch cohort (Supplementary Table 5).

Following a fixed effects meta-analysis of all the discovery and replication results, two regions reached a $P_{combined} < 5x10^{-8}$ (Table 1). These were on chromosome 6p21, with the top SNP rs9257809 ($P_{combined} = 4.30x10^{-9}$), and chromosome 16q24, with the top SNP rs9936833 ($P_{combined} = 2.68x10^{-9}$) (Figure 2). Details of the other SNPs for which replication was attempted but further evidence is required can be found in Supplementary Tables 4 and 5.

Table 1. Loci newly associated with risk of Barrett's Esophagus. Discovery and replication results at the lead SNPs at the two loci for which there is combined evidence at $P < 5x10^{-8}$. Discovery and Combined P values are two-sided, Replication P values are one sided in the direction of risk in the discovery. 'RAF'- Risk allele frequency.

				Discovery				Replication		
				Cases	Controls			UK	Dutch	
Chr	rsID	Position	Risk allele	RAF	RAF	P value	OR (95% CI)	P value	P value	P Comb
6	rs9257809	29464310	A	0.902	0.872	$2.94x10^{-7}$	1.36 1.21-1.53)	0.0361	$6.89x10^{-4}$	$4.30x10^{-9}$
16	rs9936833	84960619	C	0.418	0.375	$7.84x10^{-6}$	1.20 1.11-1.29)	$2.37x10^{-5}$	0.207	$2.68x10^{-9}$

We performed tests for pair-wise interaction (see Supplementary Methods) in the discovery data between all pairs of the 16 SNPs which were typed in the Dutch replication data (Supplementary Table 5), but no significant interactions ($P<0.01$) were found.

The SNP rs9936833 maps 24kb from the spliced, non-coding transcript LOC732275. The closest coding gene, 141kb towards the telomere, is *FOXF1*, a forkhead family transcription factor that acts in the hedgehog signaling pathway. FOXF1 is known to have a role in the development of the gastrointestinal tract and has been reported to cause esophageal structural alterations especially esophageal atresia when inactivated [18]. The region around rs9936833 contains multiple binding sites for specific transcription factors, such as FOXP2, that are known to control *FOXF1* expression (http://genome.ucsc.edu/encode).

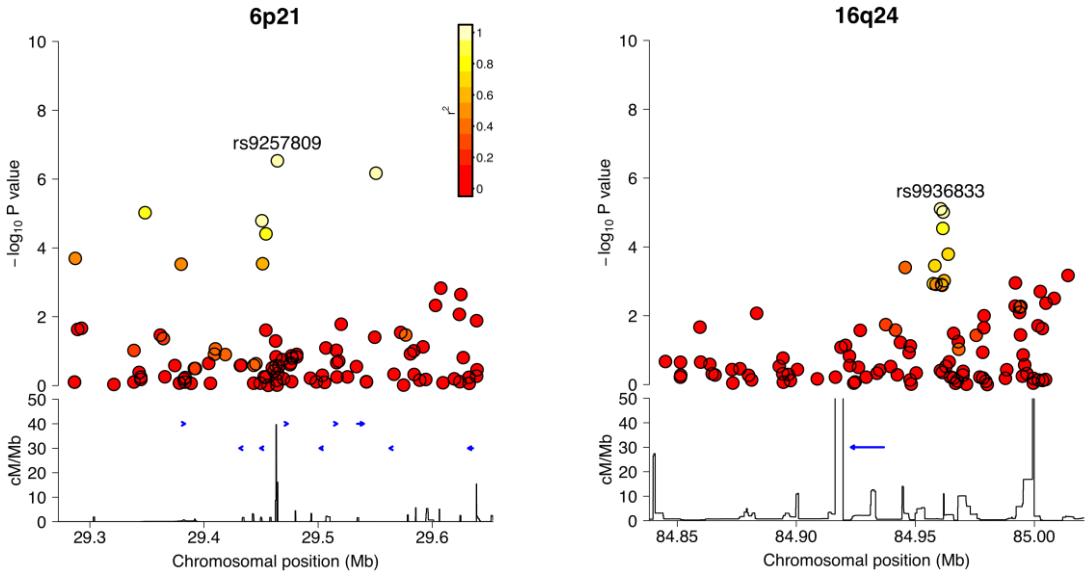

Figure 2. Regional association plot of the associated loci as detailed in Table 1, showing the signal at the lead SNP. The −log10 P values for the SNPs are shown on the upper part of each plot. SNPs are colored based on their r^2 with the labeled hit SNP which has the smallest P value in the region. r^2 is calculated from the 58C data. The bottom section of each plot shows the fine scale recombination rates estimated from individuals in the HapMap population, and genes are marked by horizontal blue lines.

The SNP rs9257809 lies on the telomeric edge of the major histocompatibility complex (MHC) region (chromosome 6p22.1: 29,587,512-33,516,520 bases) between olfactory receptor genes *OR2D12* and *OR2D13.* It is in strong long-range linkage disequilibrium (r^2>0.6 calculated in the control data) with SNPs over 1 megabase away, including two at which replication was attempted; rs13211507 ($P_{combined}=1.43x10^{-8}$) and rs9262143 ($P_{combined}=6.61x10^{-8}$). When conditioning on rs9257809, no other SNP in the MHC was significant at $P<10^{-5}$.

To further investigate the SNP signal in the MHC region we took two approaches; GENECLUSTER, which is a Bayesian tree building method [19,20] and HLA*IMP, which is a method for imputing classical HLA alleles from SNP data [21]. Both methods found evidence of association for a protective effect conferred by three HLA alleles that are in strong LD with each other (HLA-C*07:01, HLA-A*01:01 and HLA-B*08:01), see Supplementary Table 6 for HLA association results. However, conditional analysis suggests the SNP signal is a better tag for the association and none of these three classical HLA alleles show a signal in the replication data.

To investigate whether the BE SNPs are associated with expression quantitative trait loci (eQTLs), we queried the online databases Genevar [22] and SCAN [23,24]. The top SNP rs9257809 and others correlated with it are eQTL for a number of genes in a number of cell types including HLA-G in lymphoblastoid cells. However, current

eQTL databases are limited to specific populations and cell types, and the cell types included at this time are not known to be directly related to BE.

As well as case-control status, data were available for the related BE quantitative traits of circumferential extent (C) and maximal extent (M) of the length of Barrett's segment. In the discovery cohort the C measurement was available for 1,744 cases, and the M measurement for 1,618 cases. In a linear regression analysis no SNPs reached $P<10^{-6}$ in the analysis of C. One SNP (rs1023313) reached $P<10^{-6}$ in the analysis of M, but this association did not replicate in the UK or Dutch replication sets (see Supplementary Table 7). Evidence for association with C and M at the two SNPs showing replicated association with case-control status, was P=0.63 and P=0.87 for rs9936833 and P=0.10 and P=0.09 for rs925809.

There is an established gender bias in BE susceptibility, with men at greater risk than women [3,25]. The ratio of males to females is 4:1 in our case discovery data. To see if there is a genetic basis to this gender bias we performed a sex-stratified analysis for the 16 SNPs that were taken forward into the Dutch replication (Supplementary Table 8). For the SNP showing the most evidence for a sex-specific effect from the combined discovery and replication data (rs9257809), the estimated effect size in a male only analysis was OR (95%CI) = 1.38(1.25-1.53) and in a female only analysis OR(95%CI) = 1.10(0.94-1.29). The interaction term between rs9257809 and sex was $P_{combined}=0.01$.

Previous GWAS of common diseases or phenotypes have found evidence for a model where many common variants of small effect influence risk [26,27]. We tested for these *en masse* effects in BE using two methods (see Supplementary Methods). Firstly, taking the top K SNPs in independent regions in the discovery data, we performed a sign test to see whether there was an excess (over the null value of 0.5) of SNPs for which the effect was in the same direction in the UK replication data. Secondly a disease-score test analysis was undertaken, as described by the International Schizophrenia Consortium [26]. Both methods found evidence of an excess of SNPs that have the same risk allele in both cohorts. The strongest evidence in the sign test was for the top 1,150 SNPs, $P_{uncorrected}=1.21x10^{-5}$ (Supplementary Figure 1). From the disease-score analysis the strongest evidence was for the top 1,640 SNPs, $P_{uncorrected}=8.83x10^{-12}$ (Supplementary Figure 2).

There is a well-established link between BE and obesity [28,29]. To investigate whether this may in part reflect genetic effects, we repeated the sign test at 40 of the SNPs that have been found to be associated with Body Mass Index (BMI) or Waist Hip Ratio (WHR), where genotype data or tag SNPs were available in our discovery samples [30-34]. In our data a total of 29 out of 40 BMI/WHR-associated SNPs (14 genotyped, 15 tagging, Supplementary Table 9) share the same risk alleles in BE as they do for BMI/WHR ($P = 3.21x10^{-3}$).

Our results provide direct evidence that BE etiology has a genetic component. Inference as to the underlying genes must be cautious, especially for the variant (tagged by rs9257809) in the gene-rich MHC region in which linkage disequilibrium is long-range and complex. However, the location of the other BE associated SNP, rs9936833, near *FOXF1* points to a role for structural factors in the esophagus and stomach as a disease-predisposing factor, consistent with the evidence that changes such as hiatus hernia are known to be strongly associated with BE. While rs9257809 and rs9936833 SNPs alone don't have sufficient power to predict which patients with GERD will get BE in the clinic they do point to key inherited predisposition in both altered esophageal structure and inflammatory regulation. We also found evidence to show that body weight SNPs are associated with BE, so genetic effects may in part underpin the epidemiological observation that BMI is a risk factor for BO Since BE patients have an elevated death rate from cardiovascular disease this also fits the epidemiology [35]. Given BE's accepted status as a precursor lesion, the BE SNPs that we have identified are *de facto* risk factors for EAC and may give clues as to the joint genetics behind the biology of these phenotypes.

ACKNOWLEDGMENTS

We acknowledge the patients who participated in the study and the physicians who helped in recruitment. Funding for this study was provided by the Wellcome Trust Case Control Consortium 2 project (085475/B/08/Z and 085475/Z/08/Z) and the Wellcome Trust (072894/Z/03/Z, 090532/Z/09/Z and 075491/Z/04/B). P. Donnelly was supported in part by a Wolfson-Royal Society Merit Award. We thank S. Bertrand, J. Bryant, S.L. Clark, J.S. Conquer, T. Dibling, J.C. Eldred, S. Gamble, C. Hind, A. Wilk, C.R. Stribling and S. Taylor of the Wellcome Trust Sanger Institute's Sample and Genotyping Facilities for technical assistance. We also thank Jeff Barrett for design of the Immunochip array. We acknowledge use of the British 1958 Birth Cohort DNA collection funded by the Medical Research Council (grant G0000934) and the Wellcome Trust (grant 068545/Z/02), the UK National Blood Service controls funded by the Wellcome Trust and the People of the British Isles collection, funded by the Wellcome Trust. This work was also supported by the Esophageal Adenocarcinoma GenE consortia incorporating the ChOPIN project (grant C548/A5675) and IPOD project (grant MGAG1G7R), Cancer Research UK; (ASPECT grant - C548/A4584 and D9612L00090, HANDEL grant C548/A9085), Astrazeneca UK educational grant; University Hospitals of Leicester R and D grant ; AspECT - T91 5211 University of Oxford grant- HDRMJQ0.

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CHAPTER 8

BILE ACID STIMULATED EXPRESSION OF THE FARNESOID X RECEPTOR ENHANCES THE IMMUNE RESPONSE IN BARRETT ESOPHAGUS

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Am J Gastroenterol 2008;103:1510-6

ABSTRACT

Background and aim: Barrett's esophagus (BE) is a premalignant condition of the esophagus. It is a consequence of mucosal injury from chronic gastroesophageal reflux, in which bile acids are an important toxic component. The Farnesoid X Receptor (FXR) is a nuclear receptor involved in regulation of bile acid synthesis, transport, and absorption. FXR activation is also involved in the induction of the innate immune response. This suggests that FXR is involved in the pathogenesis and inflammation seen in BE.

Methods: mRNA levels of FXR and the FXR regulated genes; ileal bile acid binding protein (IBABP), small heterodimer partner (SHP), and chemokines IL-8 and macrophage inflammatory protein 3α (MIP3α) were determined by RT-PCR. Protein expression was determined by immunohistochemistry.

Results: FXR was not expressed in squamous epithelium of healthy subjects (n=7), but was present in both squamous and columnar epithelium of BE patients. Compared to the squamous epithelium of BE patients their columnar epithelium displayed a 2.3-fold ($p=0.02$) increase in FXR mRNA. Also IBABP (2.2-fold; $p=0.0029$), SHP (2.7-fold; $p=0.007$), IL-8 (1.5-fold; $p=0.04$), and MIP3α (1.7-fold; $p=0.019$) transcription were increased. Exposure of esophageal cell-line TE7 to deoxycholic acid resulted in a similar induction. Induction was abolished by the FXR antagonist guggulsterone.

Conclusion: Expression of the bile acid receptor FXR, the bile acid metabolism genes IBABP and SHP and the chemokines IL-8 and MIP3α, are increased in Barrett's epithelium. The *in vitro* induction of FXR by deoxycholic acid suggests that bile acids can actively induce the inflammatory response in BE by recruiting immune cells.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition of the esophagus that is characterized by the replacement of normal squamous epithelium by specialized intestinal epithelium containing goblet cells. Although BE itself is an asymptomatic condition, it is associated with an increased risk for the development of esophageal adenocarcinoma (EAC) [1]. Although the exact pathogenesis is unknown, BE is thought to develop from gastro-esophageal reflux disease, and is almost always accompanied by a chronic inflammation of the esophageal lining [2].

Epithelial cells play an important role in the induction of inflammatory responses. The exposure to refluxate components such as gastric acid and bile stimulates epithelial cells to produce chemokines that promote the influx of immune cells, and thus induce an inflammatory response [3-4]. Recently we have shown that exposure of epithelial cells to certain bile acids induces the production of the chemokines MIP3α, and IL-8 [5]. However the exact molecular pathways that are involved in the expression of these chemokines remains unclear.

The Farnesoid X receptor (FXR) is a nuclear receptor that regulates the expression of bile acid synthesis, transport, and absorption. The main role of FXR is to limit intracellular bile acid overload and toxicity, by acting as a bile acid sensor that activates export and absorption mechanisms [6-8]. In accordance with these functions FXR is highly expressed in the liver and small intestine. Recently FXR was found to be present in BE but not in normal squamous epithelium of the esophagus [9], the authors show a role of FXR in enhanced apoptosis seen in BE indicating that FXR has a functional role in pathobiology of BE. The functional expression of FXR in BE is combined with a recent report that FXR is also expressed by human immune cells [10], suggests a role for FXR in the inflammatory response observed in BE, e.g. by its action on transport of bile acids into Barrett's epithelial cells and a stimulatory effect on the inflammatory response. This prompted us to test for the putative role of FXR in the initiation and maintenance of the inflammatory response in BE. In order to test this we have compared the relative expression levels of FXR and two important FXR regulated bile acid metabolism factors, i.e., small heterodimer partner (SHP) and ileal bile acid binding protein (IBABP) [6-8, 11] and the chemokines IL-8, and the macrophage inflammatory protein 3α (MIP3α) in normal healthy squamous epithelium and in cylindrical Barrett's epithelium. These two chemokines have been selected as they are important promoters of the influx of neutrophils (IL-8) and B-cells (MIP3α) [12], cells that are abundantly present in BE [13]. Our findings confirmed that bile acid can indeed induce the transcription of these genes as exposure of the esophageal TE7 cell line to bile acids not only affect the transcription of FXR and IBABP but also induces IL-8 and MIP3α transcription. This chemokine induction was

absent when cells were exposed to bile acids in the presence of the FXR inhibitor guggulsterone [14-16].

MATERIALS AND METHODS

PATIENTS

Patient characteristics are outlined in Table 1. Paired biopsy samples were collected from 15 patients with a known history of BE. As controls, we collected paired esophageal biopsies from seven control subjects who underwent endoscopy for an unrelated cause, did not display any endoscopic signs of reflux and/or Barrett's, had no reflux-related complaints, odynophagia, or problems with passage of food through the esophagus. From each BE patient, two-paired biopsy sets were obtained; two adjacent samples from normal squamous epithelium, and two adjacent samples from the Barrett's epithelium. From each healthy control, a single paired set was obtained from squamous epithelium approximately 2 cm above the squamo-columnar junction. Endoscopic evaluation, biopsy collection, and histopathologic assessment were performed using routine standard procedures. One sample of each set was used for the experimental analysis as described below, the other, adjacent sample was analyzed by an expert GI pathologist (HvD), who evaluated hematoxylin and eosin stained sections for the presence of specialized intestinal metaplasia characteristic of BE [17]. The study was approved by the local ethical review board of the Erasmus MC - University Medical Center Rotterdam and informed consent was obtained from all patients prior to endoscopy.

Table 1. Patient characteristics

	Healthy controls (n=7)	BE patients (n=15)
Age (years)	36 ± 11	63 ± 14
Male (%)	4 (57)	10 (66)
Length BE segment (cm)	-	5.2 ± 1.7
Proton pomp inhibitor medication (%)	1 (14)	11 (73)

SEMI-QUANTITATIVE RT-PCR

Total RNA was isolated from the biopsies using TRIzol-reagent (Invitrogen, Groningen, The Netherlands) and remaining traces of chromosomal DNA were eliminated using a DNA-free RNA kit (Zymo, Orange, CA, USA). cDNA was synthesized with Avian Myeloma Virus reverse transcriptase according to the instructions of the manufacturer (Promega, Madison, WI, USA) as previously described [18]. PCR reactions were performed with GoTaq (Promega) according to the

manufacturer instructions. All primers are listed in Table 2 and were designed with aid of primer designer software (Clone manager, version 8, Scientific and Educational Software, Cary, NC, USA) using sequences from the NCBI database (http://www.ncbi.nlm.nih.gov). PCR conditions were 35 cycles consisting of a 30s denaturing step at 94°C; a 30s extension step at 50-60°C (depending on primers used; see Table 2); and a 30s elongation step at 72°C. After completion of these 35 cycles a final 5 min extension step was performed at 72°C, with the exception of GAPDH for which only 25 cycles were used. PCR products were size separated on a 2% agarose gel and visualized under UV light upon staining with ethidium bromide. Band size and intensities were determined by densitometry with Kodak 1D version 3.5 software (Kodak, Rochester, NY, USA) and data were normalized using the housekeeping gene GAPDH, as described previously [18]. RT-PCR densitometric data are presented as mean ± standard error of the mean.

Table 2. PCR primers

Gene	Forward primer	Reverse primer	T[a]
FXR	5'CTGGAAGTGGAACCATACTC3'	5'GTTACAGGCATCTCTGCTAC3'	59°C
SHP	5'GGAATATGCCTGCCTGAAAG3'	5'CTCCAATGATAGGGCGAAAG3'	55°C
IBABP	5'CAGGATGGGCAGGACTTCAC3'	5' CATAGGTCACGCCTCCGATG3'	60°C
IL-8	5'GTGGCTCTCTTGGCAGCCTTCTGAT3'	5'TCTCCACAACCCTCTGCACCCAGTTT3'	55°C
MIP3α	5'ATGTCAGTGCTGCTACTC3'	5'TGTCACAGCCTTCATTGG3'	50°C
GAPDH	5'CCTGCACCACCAACTGCTTA3'	5'GCCTGCTTCACCACCTTCTT3'	56°C

[a] Temperature used for annealing these primers

STATISTICAL ANALYSES

Mann-Whitney U-test was performed to determine significant differences using SPSS v11.0 (SPSS, Chicago, Illinois, USA). A two-sided *p*-value < 0.05 was considered statistically significant.

IMMUNOHISTOCHEMISTRY

Paraffin-embedded biopsy specimens were serially sectioned at 4 μm, mounted on adhesive slides and dried overnight at 37°C. Specimens were deparaffinized, and antigen retrieval was performed for 15 minutes at 100°C in a microwave oven in 10 mmol/L Tris-EDTA buffer, pH 9.0. After cooling to room temperature, samples were blocked with non-immune serum for 20 minutes. The sections were stained using the primary antibody FXR/NR1H4 (R&D Systems, Oxon UK; clone A9033A) in a 1:100 dilution. After washing, bound antibodies were visualized with Envision (Dako B.V., Haverlee, Belgium). The sections were subsequently counterstained with Mayer hematoxylin and evaluated under a light microscope (Zeiss, Axioskop, Sliedrecht, The Netherlands). As a positive control normal human ileum and colon

was used, as negative controls we used an isotype matched primary antibody and performed stainings without the addition of the primary antibody.

CELL CULTURE

The esophageal cell line TE7 [19] was kindly provided by dr. George Triadafilopoulos, Stanford University, Palo Alto, CA., USA. This is an internationally accepted and widely used epithelial cell line as a representation of the esophagus epithelium. The cell line was cultured in RPMI1640 (pH 7.4) supplemented with 2 mM L-glutamine (Bio Whittaker, Verviers, Belgium), 10% Fetal Calf Serum (Hyclone, Logan, UT, USA), and 20 units/ml penicillin/streptomycin. Cells were routinely maintained as a subconfluent monolayer in a 75 cm^2 tissue culture flask (Greiner Bio-One) in a humidified incubator with 5% CO_2 at 37°C, for a maximum of 20 generations.

RNA ANALYSIS OF CELLS INCUBATED WITH DCA WITH OR WITHOUT GUGGULSTERONE

Cells were seeded at 0.4 x 10^6 cells/well in a 12-well plate and incubated overnight at 37°C. Subsequently, cells were exposed to different concentrations of (0-200 µM) guggulsterone (Calbiochem, VWR International, Amsterdam, The Netherlands) for 18 h, followed by incubation with deoxycholic acid (DCA) to a final concentration of 0-200 µM for a period of 0, 1, 3 and 6 h. Cells were also incubated with similar concentrations of DCA without guggulsterone using parallel wells for each time point. Prior to harvesting cells were washed once with phosphate buffered saline (pH 7.4) and lysed in TRIzol. RNA isolation and RT-PCR was performed as described above.

RESULTS

INCREASED MRNA LEVELS OF FXR AND FXR REGULATED GENES IN BARRETT'S EPITHELIUM

Figure 1 shows the relative mRNA expression level of FXR mRNA in squamous epithelium of healthy subjects (healthy patients), and paired biopsy samples of squamous and cylindrical Barrett's epithelium. Relative levels were calculated from RT-PCR data by normalization against the housekeeping gene GAPDH. No expression of FXR mRNA could be detected in esophageal squamous epithelium of healthy subjects. In contrast, in squamous epithelium of BE patients significant levels of FXR mRNA were observed. The highest levels of FXR mRNA levels were observed in the cyclindrical epithelium of the Barrett's patients, where levels were on average 2.3-fold ($p=0.02$) higher than in the corresponding squamous epithelium of these patients.

To investigate whether FXR expression in cylindrical Barrett's epithelium had a functional role, expression levels of the FXR regulated bile acid metabolism genes

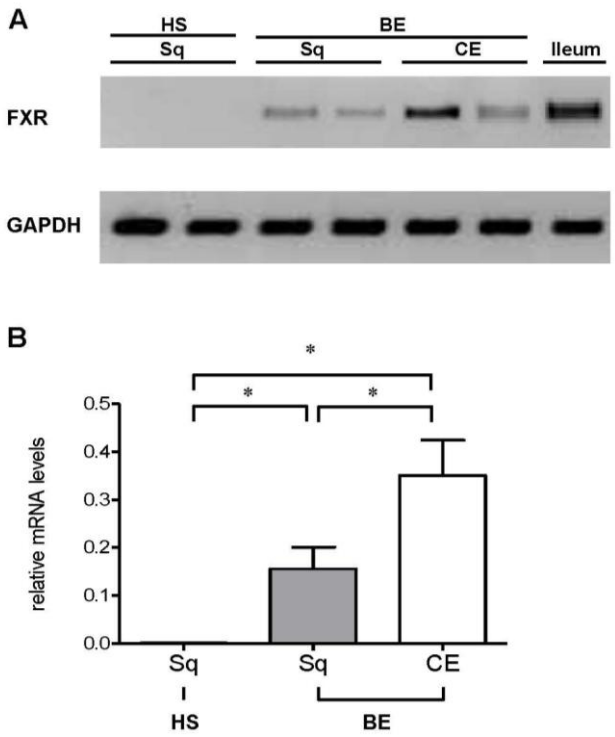

Figure 1 FXR mRNA expression in esophageal squamous epithelium (Sq) of healthy subjects (HS; n=7), and paired samples of squamous epithelium (Sq) and cylindrical epithelium (CE) from Barrett's (BE) patients (n=15), (* p<0.05). (A) Representative RT-PCR results of FXR mRNA levels in HS, 2 paired samples of Sq and BE epithelium, and as a positive control ileum. (B) Relative amounts of FXR mRNA in HS and paired samples of squamous epithelium and cylindrical epithelium from Barrett's patients. Amounts of FXR were normalized against the housekeeping gene GAPDH. The mean FXR levels values in CE were significantly higher than those seen in Sq (p=0.02).

SHP and IBABP were determined (Figure 2A). While SHP mRNA levels were below detection limit and only low levels of IBABP mRNA were observed in healthy esophageal squamous epithelium (Figure 2A), a clear signal was observed in the epithelium (both squamous and cylindrical) of BE patients. As observed with FXR also here the relative mRNA levels of SHP and IBABP were significantly higher in cylindrical Barrett's epithelium than in squamous epithelium of BE patients (2.7-fold ± 0.88 (p=0.007) for SHP and 2.2-fold ± 0.55 (p=0.0029) for IBABP).

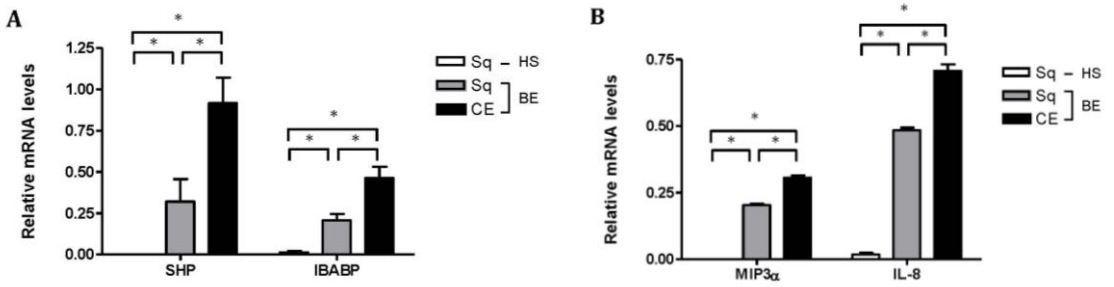

Figure 2. A) mRNA levels of SHP and IBABP in esophageal squamous epithelium (Sq) of healthy subjects (HS, n=7), and paired samples of squamous epithelium (Sq) and cylindrical epithelium (CE) from Barrett's patients (BE; n= 15), (* p<0.05). Mean mRNA values of SHP and IBABP were significantly higher in CE compared to Sq epithelium (IBABP p=0.0029; SHP p=0.007). mRNA levels were normalized against the housekeeping gene GAPDH. B) mRNA levels of MIP3α and IL-8 in esophageal squamous epithelium (Sq) of healthy subjects (HS; n=7), and paired samples of squamous epithelium (Sq) and cylindrical epithelium (CE) from Barrett's patients (BE; n=15), (* p<0.05). Mean mRNA values of MIP3α and IL-8 were significantly higher in Barrett's epithelium compared to squamous epithelium (MIP3α p=0.019; IL-8 p=0.04). mRNA levels were normalized against the housekeeping gene GAPDH.

To determine the possible role of FXR in inducing the inflammatory response, the expression levels of two important chemokines were determined. While MIP3α mRNA levels were below the detection limit and only low levels of IL-8 mRNA were observed in healthy squamous epithelium (Figure 2B), significant mRNA levels for these genes were found in the esophageal samples from BE patients. Again the highest levels for these genes were present in the cylindrical BE epithelium compared to squamous epithelium of BE patients (1.7-fold ± 0.59 (p=0.019) for MIP3α and 1.5-fold ± 0.45 (p=0.04) for IL-8).

INCREASED PROTEIN LEVELS OF FXR IN BARRETT'S EPITHELIUM

To investigate where FXR is located in various tissues and whether the observed increase in FXR mRNA levels also results in an increase of FXR protein we performed immunohistochemical stainings with an FXR specific antibody. While esophageal squamous epithelium of healthy controls did not show FXR staining (Figure 3A), both squamous epithelium (Figure 3B) and cylindrical epithelium (Figure 3D and E) of BE patients showed a strong FXR staining (Figure 3D and E). The staining was predominantly present in the nuclei of epithelial cells. Remarkably, some metaplastic regions did not show any FXR specific staining. These negative areas seemed randomly distributed throughout the crypts and were histologically indistinguishable from those that did stain. Figure 3C shows FXR staining of the ileum, which was used as a positive control.

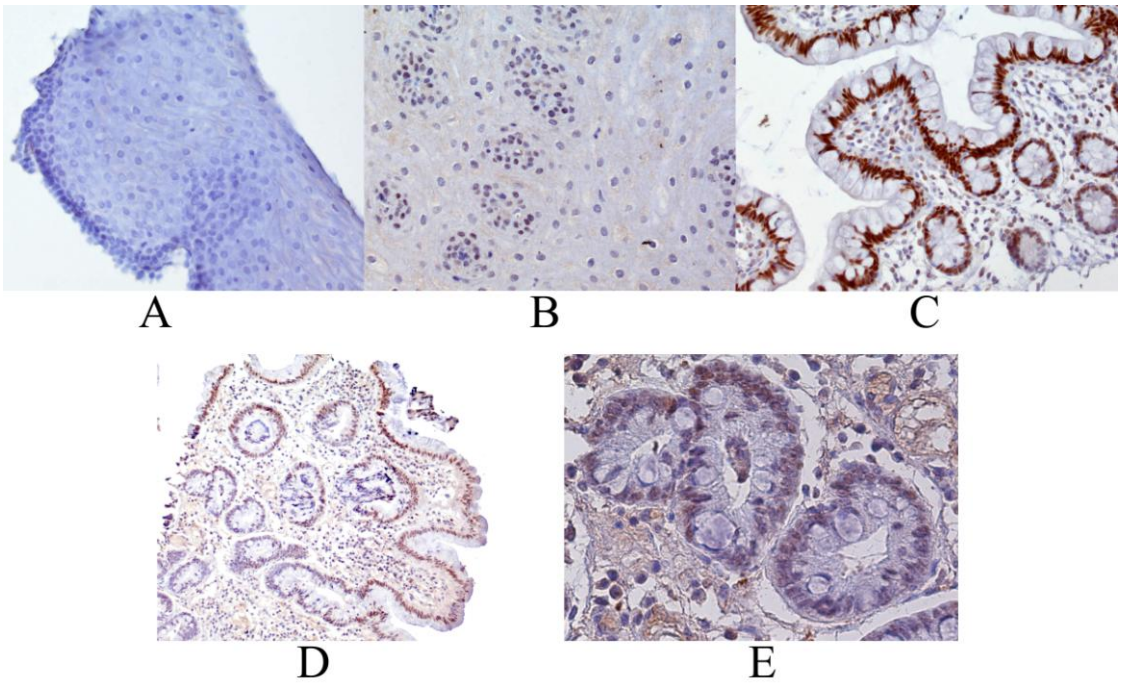

Figure 3. FXR protein expression in squamous esophageal epithelium of a healthy subject (A), squamous epithelium of a Barrett's patient (B), Barrett's epithelium (D), together with an magnification (E) and a positive control; ileum (C), visualized by immunohistochemistry using a monoclonal antibody against FXR.

DCA INDUCES IBABP AND THE CHEMOKINES IL-8 AND MIP3α IN AN FXR SPECIFIC WAY

To determine whether bile acids can activate FXR expression, TE7 cells were treated with DCA in the absence or presence of the FXR antagonist guggulsterone. A clear dose- and time-dependent increase in FXR expression levels was found after treatment with physiological levels (100-300μM) of DCA [20-22]. This response could be completely inhibited in a dose- (Figure 4) and time-dependent (not shown) manner by pre-treatment with guggulsterone.

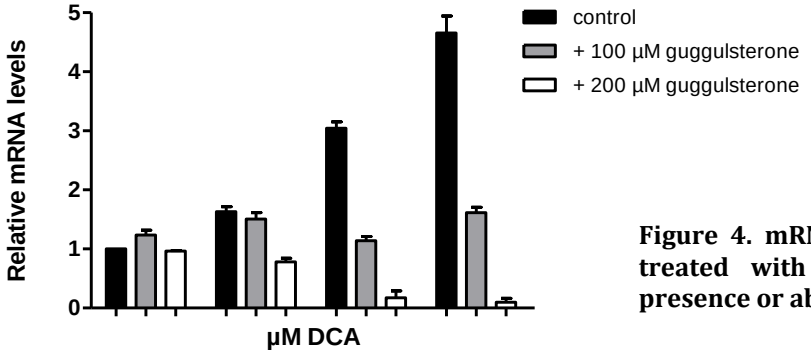

Figure 4. mRNA levels of FXR in TE7 treated with DCA for 6 h in the presence or absence of guggulsterone.

Based on these results we selected a single time point (6 h), DCA concentration (200 μM), and guggulsterone concentration (200 μM) and used these to investigate the putative involvement of FXR in the transcription of chemokines. The two chemokines IL-8 and MIP3α showed a significant increase in mRNA levels after exposure to DCA; (3.3-fold ± 0.8; p=0.007, and 19.2-fold ± 1.5; p<0.0001, respectively). Pre-treatment with guggulsterone resulted in a decreased ability of DCA to stimulate IL-8 (2.9-fold ± 0.3; p=0.005) and MIP3α (3.0-fold ± 0.8; p=0.0002) (Figure 5C and D). The mRNA levels of FXR increased 4.7-fold ± 0.5 (p=0.002) as a result of DCA treatment (Figure 5A). No DCA mediated increase in FXR levels was observed (p=0.001) when the cells had been pre-treated with guggulsterone. As a control of FXR specific activity we investigated the transcription of IBABP. The DCA mediated induction of FXR was indeed accompanied by a 1.9-fold ± 0.4 (p=0.017) increase in the expression of IBABP (Figure 5B), which was reduced upon guggulsterone treatment (1.4-fold ± 0.3; p=0.148).

DISCUSSION

BE is a premalignant condition resulting from chronic gastroesophageal reflux. Bile acids play an important role in the development of BE, since bile acids are an important toxic component of the refluxate. The nuclear bile acid receptor FXR is involved in the regulation of bile acids by regulating the synthesis, absorption and excretion of bile acids. Given the fact the FXR is also expressed on various immune

cells, where it is thought to be involved in the activation of the immunological processes [10, 23], we postulated that FXR plays a role in the pathogenesis and the inflammation seen in BE. We therefore tested the expression levels of genes involved in the bile acid metabolism (i.e. FXR) and the immune system, (i.e. IL-8 and MIP3α), in biopsies from control subjects and BE patients. The observed mRNA levels indicate a role for FXR in the induction of chemokines, which was supported by *in vitro* assays.

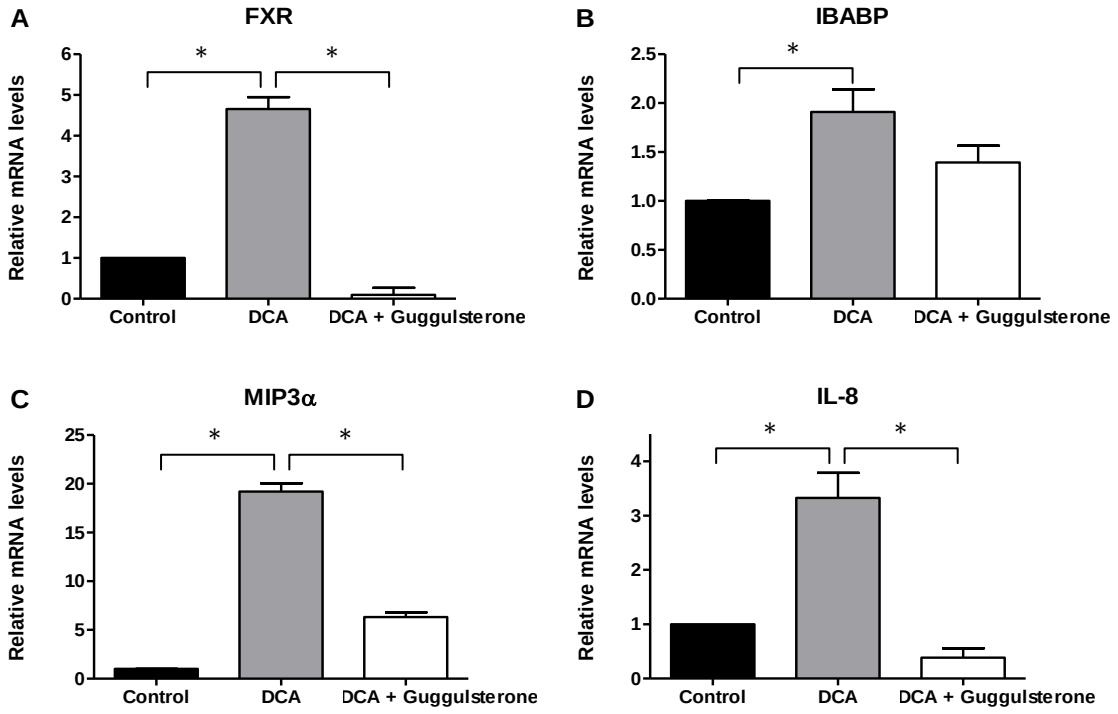

Figure 5 TE7 cells treated with DCA in the presence or absence of guggulsterone. mRNA levels of FXR (A), IBABP (B), MIP3α (C) and IL-8 (D) were determined, control levels were set on 1. The mean mRNA levels of genes increased significantly after DCA exposure compared with unexposed cells. When the cells were pre-treated with guggulsterone, an inhibitor of FXR, all genes showed a clear decrease in mRNA levels. mRNA levels were normalized against the housekeeping gene GAPDH, * $p<0.05$.

In the present study we did not observe FXR expression in the healthy esophagus, a clear expression was found in the esophageal lining of BE patients, both in the squamous epithelium and in the cylindrical BE epithelium. These results confirm those of a recent study, in which an increased FXR expression was found along with progression from normal esophagus towards BE [9]. While these authors reported a low FXR expression in normal esophagus, we could not detect any FXR above the detection level in our healthy controls. This discrepancy may be explained by the fact that the esophageal samples in the previous study were actually obtained from patients with reflux symptoms [9], while our controls did not have any signs of reflux at all, indicated by the limited use of proton pump inhibitors in the control group

(Table I). This suggests that reflux induces the expression of FXR in healthy squamous epithelium, which is in line with our findings that squamous epithelium of patients with BE and squamous epithelium of patients with reflux esophagitis (data not shown) FXR is expressed, albeit at low levels. The samples for immunohistochemistry used in this study were from routine patient materials and were not controlled. We therefore used these data primarily to provide an indication as to where FXR is located in the various tissues and if protein expression levels are increased.

A relationship between FXR expression and reflux is further confirmed by our *in vitro* studies, which show that exposure to DCA results in increased FXR expression, and FXR specific induction of chemokines. These experiments can however be criticized by our experimental set up, in which a relative long-term exposure to guggulsterone was required, and the fact that (in order to ensure cell viability), these experiments had to be performed at a neutral pH. Obviously, in patients with gastro-esophageal reflux, the distal esophagus is exposed to refluxate for short periods of time (minutes) and this occurs in a acidic environment [24]. This drawback could be overcome in future studies where the role of FXR is tested in an animal model for the development of BE [25], using FXR KO mice [26].

That the increase in FXR levels does have a physiological role in BE is supported by the fact that target genes of FXR involved in cellular bile acid metabolism, i.e., SHP and IBABP, both were increased in squamous epithelium and in Barrett's epithelium of BE patients (Figure 2). This implies the presence of a functional bile acid transport system, suggesting an adoption of the esophagus to the potentially toxic exposure of bile acids in BE patients.

On the other hand the ectopic expression of FXR might well enhance the inflammatory reaction seen in BE patients. These chemokines have been shown to promote the influx of immune cells, such as neutrophils (IL-8) and B-cells (MIP3α) [12]. Both these chemokines (Figure 2) and cell types [13] are abundantly present in BE. In addition, our *in vitro* experiments clearly showed that exposure to DCA resulted in a significant increase in mRNA levels of IL-8 and MIP3α (Figure 5). This increase was inhibited by guggulsterone, a specific inhibitor of FXR. It is understandable that guggulsterone inhibits the synthesis of mRNA for genes regulated by FXR, but this same pattern was unexpectedly also observed for the mRNA levels of FXR itself. Nevertheless our results suggests a direct involvement of FXR in the chronic inflammation observed in BE patients.

Our results suggest an important role for biliary reflux in the initiation and maintenance of the inflammatory response in Barrett's esophagus. Additionally, a pathogenic role of FXR in the development of BE towards adenocarcinoma is supported by the recent findings that the FXR antagonist guggulsterone also leads to

an increase in apoptosis [9]. FXR could therefore be a potential target for future therapies preventing the development of BE and esophageal adenocarcinoma.

ACKNOWLEDGEMENT

The authors would like to thank dr. A.H.M. van Vliet for helpful and stimulating discussions. Furthermore we would like to thank the people of the endoscopy unit at the Erasmus MC for their assistance with obtaining the biopsies.

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CHAPTER 9

GENERAL DISCUSSION AND SUMMARY

ALGEMENE DISCUSSIE EN SAMENVATTING

Anouk van de Winkel

GENERAL DISCUSSION AND SUMMARY

Many studies have attempted to elucidate the etiology of multifactorial Barrett esophagus in attempting to discover new targets for treatment of this precancerous lesion and its sequela esophageal adenocarcinoma. Few appear to have found genes with a true causative function, others discover genes resulting from concomitant processes induced by cell stress, inflammation and differentiation or from epiphenomena. Nonetheless, the latter may be useful as marker or chemopreventive targets in neoplastic progression. The new insights emerging from the findings of our work are threefold. We start with discussing the importance and role of nuclear receptors in BE and grading dysplasia. After these pathologic findings, we evaluate observations from genetic studies rendering new insights in predisposition of BE and EAC, and lastly we touch upon acquired knowledge of the molecular mechanism responsible for the manifestation of BE.

The work presented in this thesis, firstly focused on nuclear receptors. The nuclear receptor Pregnane X Receptor (PXR) is an important factor in the detoxification of xenobiotics and bile acid homeostasis and was investigated for its expression and genetic variation in esophageal disease. Expressing highly in the columnar esophageal epithelium of BE patients and tumor tissue of EAC patients, PXR appears to be activated upon bile acid stimulation. Together with the observation of a BE associated PXR SNP, PXR is hypothesized to have a function in predicting progression and treatment of esophageal disease. Next, we sought out to study the expression and localization of PXR further in biopsies taken at different stages of neoplasia and esophageal adenocarcinoma. Interestingly, from these results we learned that PXR is able to separate high-grade dysplasia from low grade or non-dysplastic tissue. Additionally, our observations implied a possible field effect of PXR expression in squamous epithelium of adenocarcinoma patients. Clinical trials will have to examine if PXR expression can be detected in patients with reflux esophagitis and Barrett's esophagus, or possibly even in oral samples from the general population. Future studies will show if PXR possesses this predictive power crucial for identifying subjects or patients at risk for developing esophageal adenocarcinoma. In addition to PXR, a second nuclear receptor, namely the bile acid receptor Farnesoid X Receptor (FXR) was examined in the before mentioned immunohistochemical study. Previously, we learned that FXR is important in the immune response in BE, by attracting neutrophils and B-cells via production of the chemokines IL-8 and MIP3α. Notably, our work proposes decision chart that offers the combination of nuclear PXR and FXR expression for use as a diagnostic tool. Together the results from these studies encourage future research to investigate the role of nuclear receptors PXR and FXR in Barrett's esophagus and to further evaluate their potential clinical value in improving the accuracy of grading dysplasia and predicting progression.

Secondly, the presented work reports an increased risk of BE and esophageal adenocarcinoma in individuals lacking copies of the 1633G/1453T haplotype of the vitamin D receptor gene. This protective haplotype is suggested to bind transcription factor GATA-1 resulting in a decreased vitamin D receptor expression and thus an impaired vitamin D signaling. This exposes possible detrimental effects of vitamin D with respect to the development of esophageal adenocarcinoma or its precursor BE. This especially deserves notice as vitamin D is generally considered to be protective against solid tumors. Additional research is obviously needed to confirm the aforementioned association and to clarify the mechanisms by which vitamin D affects the risk of esophageal disease, and even more importantly to investigate how serious this impact of vitamin D truly is in patients with BE. Therein, obesity is an important factor to take in account. We hypothesize that the development of an esophageal adenocarcinoma, which often goes hand-in-hand with rapid weight loss, may cause fast liberation of excessive amounts of vitamin D from its adipose storage. This emphasizes why our findings that the adverse effects of vitamin D may be underestimated are so indispensible and encourages the further identification and refinement of cohorts of subjects most likely to benefit from chemopreventive agents. At the same time, evidence from ecological studies mimicking true long-life effects of vitamin D may endorse the idea of personalized recommendations for vitamin D supplementation.

In search for other predisposing genes, the first genome wide association study on BE performed is described in this thesis. Single nucleotide polymorphisms in two regions were associated with BE risk; one within the major histocompatibility complex, and one close to FOXF1, which is implicated in esophageal development. Observations in this genetic study provide direct evidence that BE etiology indeed has a genetic component, which is mediated by many common variants of small effect. The well-established link between BE and obesity lead to further analysis, revealing that the risk alleles for polymorphisms predisposing to obesity are also associated with an increased risk of BE. This leads to believe that genetic effects may in part underpin the epidemiological observation that body mass index is a risk factor for BE.

The final development emerging from the work presented in this thesis involves key events that may lead to genetic transdifferentiation observed in BE. It is intriguing that HOXA genes were shown to be upregulated with high correlation to mucin expression. Jointly with the notion that bile acids activate certain HOXA genes, this indicates that reprogramming of enteric HOX code may truly contribute to molecular causation of BE. Remarkably, this was found to be independent of CDX2, which may be of subordinate importance in the true causative events that result in BE.

The precursor cell from which Barrett's esophagus develops has yet to be determined and studies have shown that intestinal metaplasia of esophageal

squamous epithelium involves more than one cellular pathway. Regardless of this, bile acids seem to be a necessary trigger therein, and as they bind nuclear hormone receptors PXR and FXR, which are both powerful transcriptional regulators, these may well the mediators of the observed bile acid-induced effects on HOX coding. In this artwork, we have also incorporated putative tools and targets for diagnosis and treatment of Barrett's esophagus before full-blown adenocarcinoma evolves, from findings presented in this thesis (see Figure 1, page 144).

ALGEMENE DISCUSSIE EN SAMENVATTING

De behandeling en vooruitzichten voor patiënten met een slokdarm carcinoom zijn weinig hoopgevend. Vroegtijdige detectie en preventieve behandelingen zijn noodzakelijk, en daarom richt veel onderzoek zich op de voorloper aandoening Barrett slokdarm, waarin gezond slokdarm 'epitheel' (bovenste laagje cellen) vervangen is door darmachtig epitheel. De precieze ontstaanswijze van een Barrett slokdarm en de daaropvolgende 'progressie' (het verdere verloop naar dysplasie stadia en het uiteindelijk adenocarcinoom van de slokdarm) is echter grotendeels onduidelijk. Veel studies hebben getracht hier meer inzicht in te krijgen om nieuwe handvaten te ontdekken voor vroegtijdige behandelingen. Het is bekend dat een Barrett slokdarm ontstaat als gevolg van een combinatie van meerdere factoren, waarvan chronische oprisping van gal en zuur uit de maag één van de voornaamste is. Slechts enkele lijken genen gevonden te hebben die daadwerkelijk een Barrett slokdarm kunnen veroorzaken, anderen hebben genen gevonden die slechts een gevolg zijn van bijverschijnselen. Desondanks kunnen laatstgenoemde genen bruikbaar zijn als markers of als aangrijpingspunten voor chemotherapie voorkomende behandelingen in de ontwikkelingsgang naar kwaadaardige stadia. Een algemene inleiding is te vinden in Hoofdstuk 1. Hoofdstuk 2 is een verslag van de "Digestive Disease Week 2011", een wereldwijd congres dat jaarlijks gehouden wordt in de Verenigde Staten, en omvat een overzicht van recente bevindingen in het veld van klinisch en basaal slokdarm onderzoek.

De nieuwe inzichten die uit dit proefschrift naar voren komen zijn drieledig. Ten eerste beschrijven we het belang en de rol van nucleaire receptoren in de Barrett slokdarm en in het stageren van het verloop hiervan. Naast deze pathologische bevindingen evalueren we observaties van genetische studies, die nieuwe inzichten gaven in welke personen het meest ontvankelijk zijn voor een Barrett slokdarm of slokdarmkanker. Uiteindelijk bespreken wij verworven kennis van het moleculaire mechanisme dat verantwoordelijk is voor het ontstaan van een Barrett slokdarm.

In de eerste plaats richt het onderzoek beschreven in dit proefschrift op nucleaire receptoren, eiwitten in de kern van cellen die allerlei lichaamsprocessen aansturen. De nucleaire receptor ´pregnane X receptor´ (PXR) is een belangrijke factor in het ontgiften van lichaamsvreemde stoffen en in het behouden van een goede galzuurbalans. PXR komt sterk tot expressie in de slokdarm cellen van Barrett patiënten en in tumor weefsel van slokdarmkankerpatiënten. Het lijkt geactiveerd te worden doordat het galzuren aan zich bindt. Samen met de waarneming dat een specifieke mutatie in PXR verband houdt met hebben van een Barrett slokdarm, lijdt dit tot de veronderstelling dat PXR een functie heeft in het voorspellen van het verloop en de behandeling van slokdarmziekte aandoeningen (Hoofdstuk 3). Vervolgens bestudeerden we de aanwezigheid en lokalisatie van PXR, door hiervoor een specifieke kleuring te doen op biopten (kleine hapjes weefsel) genomen uit de

slokdarm in verschillende stadia van dysplasie en met een adenocarcinoom van de slokdarm. Interessant is dat we uit deze resultaten hebben geleerd dat PXR in staat is om hooggradige dysplasie (grote kans op slokdarmcarcinoom) te onderscheiden van laaggradig of niet-dysplastisch weefsel (kleine kans op slokdarmcarcinoom) (Hoofdstuk 4). Bovendien impliceren onze waarnemingen een mogelijk "veld effect", omdat we expressie van PXR vinden in het gezonde weefsel direct aangrenzend aan de tumor van slokdarmkankerpatiënten. Klinische studies zullen moeten onderzoeken of de aanwezigheid van PXR in weefsel kan worden gedetecteerd bij patiënten met 'reflux oesofagitis' (chronische ontsteking van slokdarm veroorzaakt door oprispend gal en zuur uit de maag) en Barrett slokdarm, of misschien zelfs in orale weefselmonsters uit de algemene populatie. Toekomstige studies zullen uitwijzen of PXR deze voorspellende kracht bezit, welke van cruciaal belang zou zijn voor het identificeren van personen of patiënten met een verhoogd risico op het ontwikkelen van een adenocarcinoom van de slokdarm.

Naast PXR is een tweede nucleaire receptor, namelijk de galzuur receptor 'farnesoid X receptor' (FXR) onderzocht in dezelfde biopten als in de voorgenoemde studie. Uit een vorige studie hebben we geleerd dat FXR belangrijk is in de 'immuunrespons' (afweerreactie) in de Barrett slokdarm, door de zogenaamde chemokines IL-8 en MIP3α aan te maken die witte bloedcellen, te weten neutrofielen en B-cellen, aantrekken (Hoofdstuk 8). In het bijzonder brengt dit werk een beslismodel te berde waarin de combinatie van PXR en FXR kleuringen in celkernen bruikbaar wordt als diagnostisch hulpmiddel in Barrett patiënten (Hoofdstuk 4). De resultaten van deze studies moedigen aan om in de toekomst de rol van nucleaire receptoren PXR en FXR te onderzoeken in Barrett slokdarm; enerzijds om te kijken wat hun klinische waarde is in het verbeteren van de diagnostiek van het dysplastische verloop van een Barrett slokdarm; anderzijds om te analyseren of deze receptoren kunnen voorspellen wie een groot risico loopt op kwaadaardige progressie.

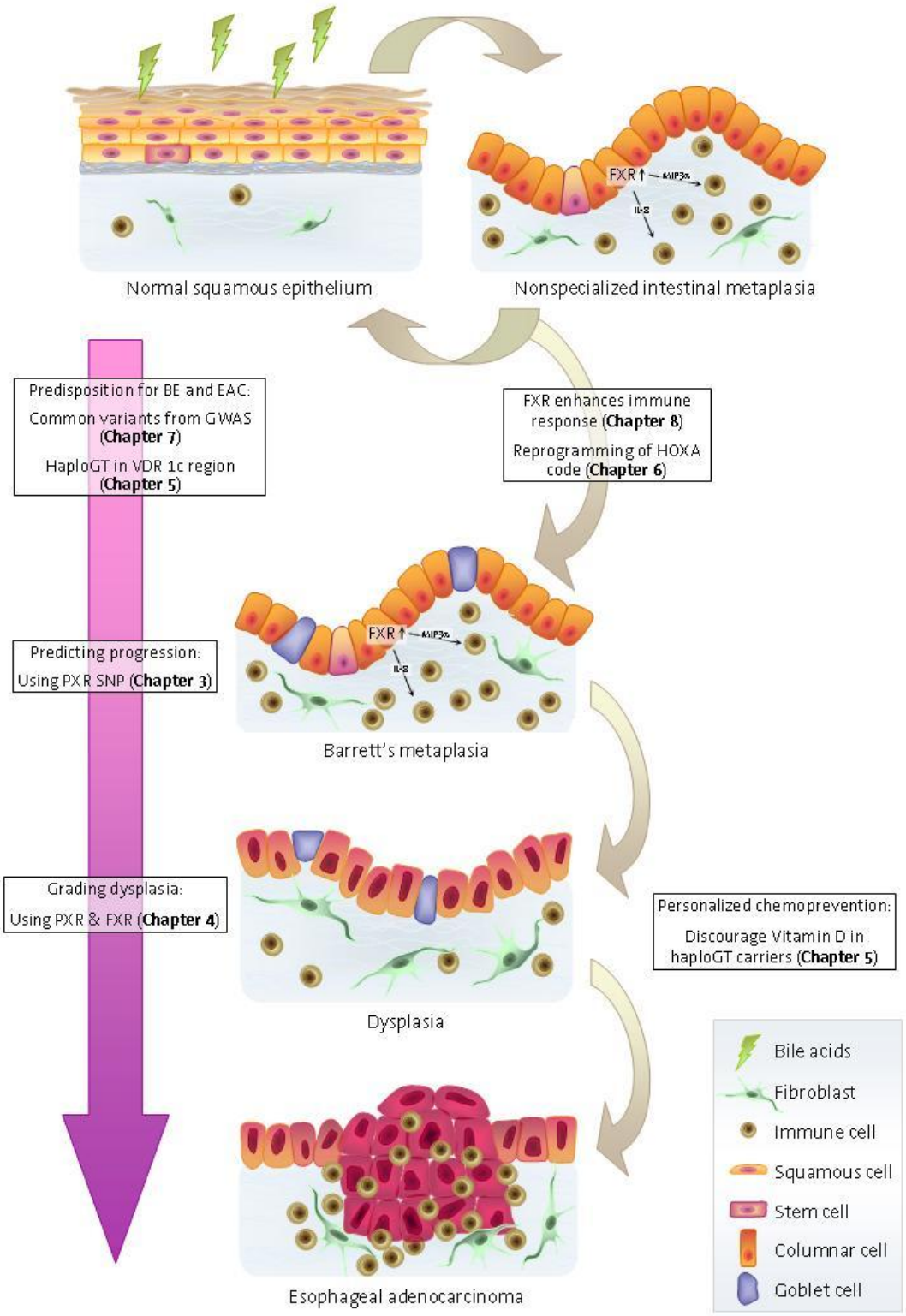
In het tweede, genetische deel, leren bevindingen uit dit proefschrift dat bij personen bij wie kopieën van het beschermende '1633G/1453T haplotype' in hun DNA ontbreekt, een verhoogd risico op een Barrett slokdarm en slokdarmkanker is geconstateerd (Hoofdstuk 5). Dit haplotype omvat vijf samenhangende DNA mutaties in een kleine regulerende regio van het Vitamine D receptor gen. Dragers van dit haplotype hebben een lagere hoeveelheid vitamine D receptor waardoor vitamine D haar biologische effecten minder goed kan doorgeven. Het feit dat deze verminderde vitamine D-signalering in onze handen beschermend lijkt te zijn voor een Barrett slokdarm en adenocarcinoom verdient aandacht, want vitamine D staat algemeen bekend als beschermend tegen kanker. Indien toekomstige studies erin slagen de patiënten die genetisch gezien het meeste baat hebben bij chemopreventieve medicatie en vitamines, zal een persoonsspecifieke behandeling zoals bijvoorbeeld een aanbeveling voor vitamine D-suppletie een realiseerbaar doel worden. Om uit te wijzen hoe ernstig het mogelijk nadelige effect van vitamine D

werkelijk is, zijn aanvullende onderzoeken nodig om onze bevindingen te bevestigen en het onderliggende mechanisme te verduidelijken.

Op zoek naar nieuwe genen die iets vertellen over de ontvankelijkheid voor slokdarmaandoeningen, laten we resultaten zien van de eerste genoom-wijde associatie studie uitgevoerd op gezonde controles en patiënten met een Barrett slokdarm of een adenocarcinoom van de slokdarm (Hoofdstuk 7). Kleine mutaties in het DNA van het gehele menselijke genoom, welk ongeveer 20.000 to 25.000 genen omvat, werden onderzocht. Dit heeft twee genen onder de aandacht gebracht die mutaties bevatten die geassocieerd blijken te zijn met het risico op een Barrett slokdarm. Hun rol in de Barrett slokdarm is op het moment nog onduidelijk en zal een brandpunt worden van actueel slokdarmonderzoek. Verder blijkt er inderdaad een genetische component aan het ontstaan van een Barrett slokdarm te zitten en deze betreft een complexe combinatie van veel voorkomende mutaties met een klein effect. Verder zijn genen gerelateerd aan obesitas, oftewel zwaar overgewicht, ook geassocieerd met een verhoogd risico op Barrett slokdarm. Dit leidt tot de conclusie dat het gevestigd feit dat obesitas een risicofactor is voor het ontwikkelen van een Barrett slokdarm ten minste voor een deel te wijten is aan een genetisch effect.

Ten slotte laten we in dit proefschrift bevindingen zien betreffende genen die direct verantwoordelijk kunnen zijn voor het ontstaan van een Barrett slokdarm. Tijdens de embryonale ontwikkeling vindt een vergelijkbaar proces plaats, waarbij de darmachtige cellen van het toekomstige maag-darmkanaal gedeeltelijk vervangen worden door slokdarmachtige cellen. Homeobox (HOX) genen spelen hierin een cruciale rol. In de Barrett slokdarm zijn sommige van deze HOX genen geactiveerd en andere gedeactiveerd, en deze herprogrammering komt overeen met de uit de embryologie bekende effecten op celtype. Verder tonen wij aan dat galzuren bepaalde HOX genen activeren. Dit suggereert dat HOX genen daadwerkelijk deel is van de moleculaire oorzaak van het ontwikkelen van een Barrett slokdarm. Aanvullende studies zijn nodig om de regulerende rol van HOX genen hierin te bestuderen.

De voorloper cel of stamcel waaruit een Barrett slokdarm zich ontwikkelt, is vooralsnog onduidelijk. Wel is vastgesteld dat galzuren de aanstichters moeten zijn en dat er meerdere cellulaire routes betrokken zijn. Uit de bevindingen van dit proefschrift concluderen wij dat zij aan nucleaire receptoren zoals PXR en FXR binden, die beide krachtige regulatoren van genen zijn, en zo HOX genen herprogrammeren met als gevolg het ontstaan van een Barrett slokdarm. Figuur 1 vat dit grafisch samen en omvat tevens aangrijpingspunten voor de diagnose en behandeling van een Barrett slokdarm, zowel voor- als nadat een volgroeid adenocarcinoom zich ontwikkelt.



Figuur 1. Schematisch overzicht van het werk dat in dit proefschrift beschreven wordt. In het hypothetische mechanisme geven galzuren continue stress aan gezonde slokdarmcellen, waardoor processen zoals FXR activatie, afweerreacties, en signalering naar stamcellen plaatsvinden. Reactivering van HOX genen resulteren in het ontstaan van de darmachtige cellen die karakteristiek zijn voor een Barrett slokdarm. Bevindingen omtrent mogelijke genetische voorspellers, diagnostische hulpmiddelen en preventieve strategieën zijn eveneens in deze illustratie opgeomen. (Aangepast uit figuur 1 van hoofdstuk 2)

SUPPLEMENTARY DATA

SUPPLEMENTARY TABLES (CHAPTER 5)

Table S1

Risk allele	BE *versus* HC OR (95% CI)	VDR mRNA level Mean (SD)	P-Value for overall trend*
HAP1GT Zero	1	0.116	
HAP1GT One	0.84 (0.54-1.30)	0.067	
HAP1GT Two	*0.46 (0.26-0.80)*	0.055 (0.021)	0.3
HAP1GC Zero	1	0.175	
HAP1GC One	*1.53 (1.02-2.30)*	0.089	
HAP1GC Two	*1.84 (1.02-3.32)*	0.078 (0.073)	0.8
HAP1CC Zero	1	0.075	
HAP1CC One	1.12 (0.74-1.68)	0.065	
HAP1CC Two	0.96 (0.41-2.21)	0.245 (0.063)	0.01

Table S2 Frequencies of haplotypes of the 1c-regulatory region of VDR gene in Whites, Asians and Africans

SNP ID	Minor Allele*	MAF (%) IN Whites (468 Chromosomes)	Asians (214 Chromosomes)	Africans (116 Chromosomes)
1c-T−1930C	C	40	34	14
1c-G−1633C	C	28#	20#	30#
1c-C−1453T	T	41#	33#	51#
1c-G−1156A	A	29	17	11#

*Minor alleles were determined on the basis of frequency in the white population.
#**VDR** tagging SNPs for association study of different ethnic groups.

SUPPLEMENTARY FIGURES (CHAPTER 5)

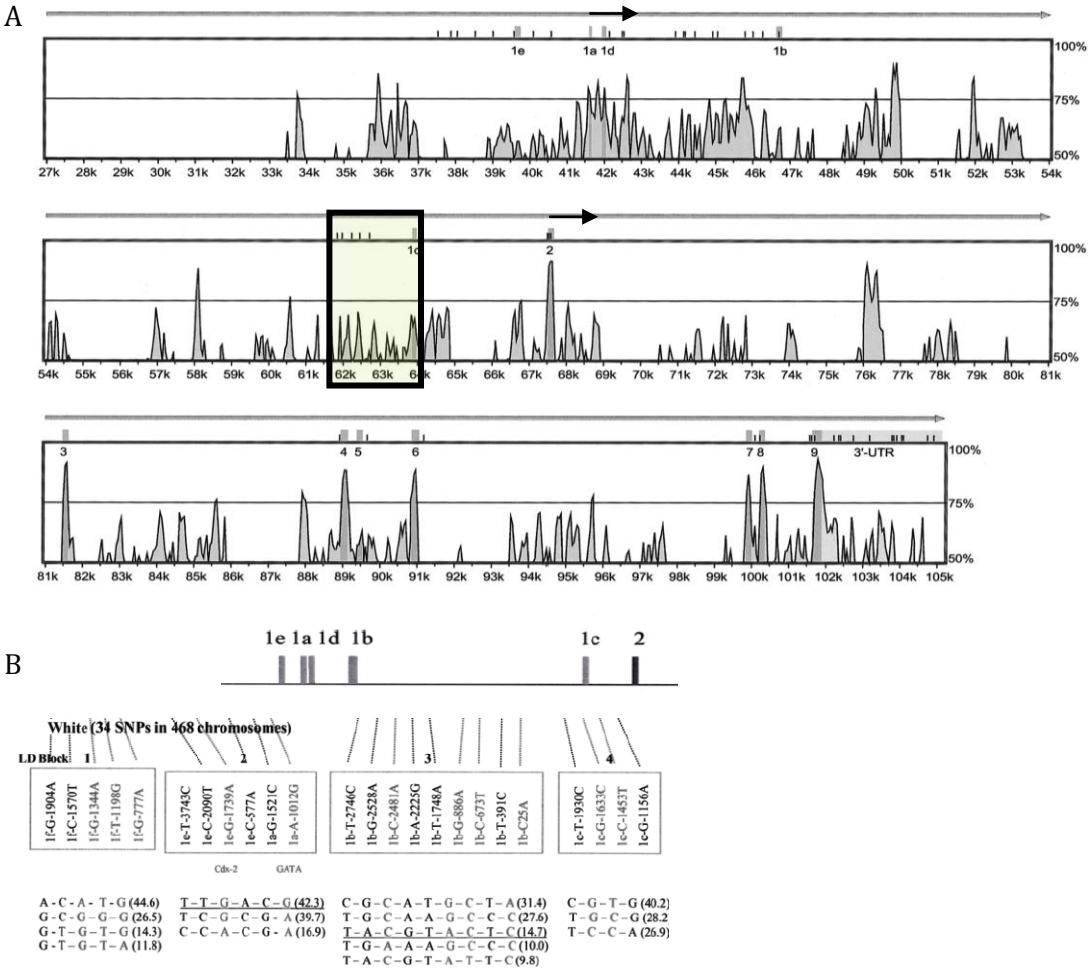


Figure S1. Genomic analysis of the 5'regulatory region of the VDR gene A) Conservation of the human and mouse genomic VDR gene sequence. The Y-axis is the homology rate between human and mouse; the X-axis is the physical distance on the human *VDR* gene. All exons are indicated in blue, the 3' UTR in light blue, and the conserved non-coding region in red. The green box indicates the VDR 1c region investigated in the current study. The small black bars on the top of each frame indicate the polymorphisms observed by Fang et al. by resequencing, and the gray arrow on top indicates the transcription direction of VDR. (Adapted from: Fang Y, Am. J. Hum. Genet, 2005) B) Position of 35 sequence variations and 15 previously determined and characterized haplotype tagging SNPs[33].

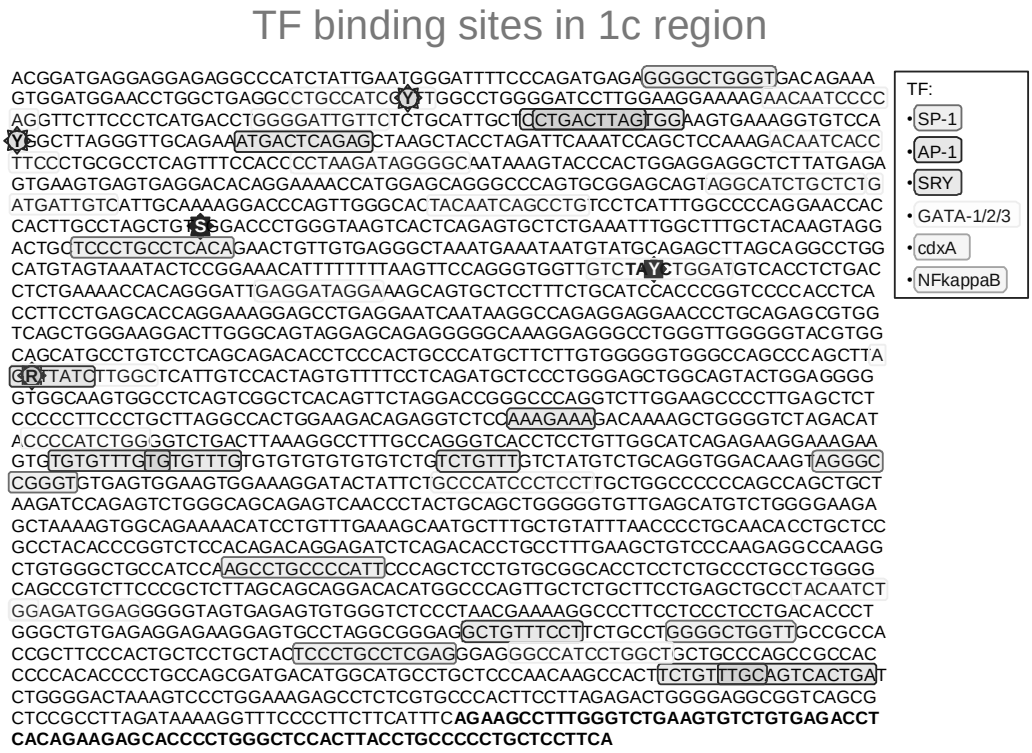


Figure S2. Potential transcription factor binding sites in 1c region of VDR gene. The intronic region 2 kb upstream of 1c exon contains a number of potential binding sites for cis elements, including SP1, AP1, SRY and GATA-1, -2 and-3, CdxA, NF-κB, and others. TRANSFAC analysis of the 5' noncoding 1c-region of VDR indicated that three htSNPs of the studied LD block change the core recognition sequence of GATA-1 transcription factor. S= G or C, Y = C or T, R = A or G according to The International Union of Pure and Applied Chemistry (IUPAC) nucleotide base code.[38] The third and fourth SNP (indicated by solid stars) represent the 1633G/C and 1453C/T SNPs that are examined in this study.

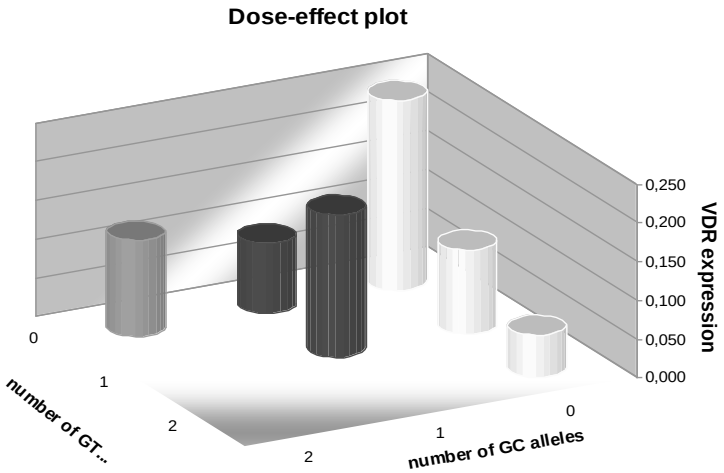


Figure S3. Dose-expression plot: Genotype-related mRNA levels of VDR in esophageal Barrett epithelium. VDR levels in esophageal epithelium of BE patients are determined by quantitative Real-Time PCR and plotted by genotype. Levels were highest in tissue from patients with two alleles of CC haplotype and lowest in patients with two alleles of the protective GT haplotype.

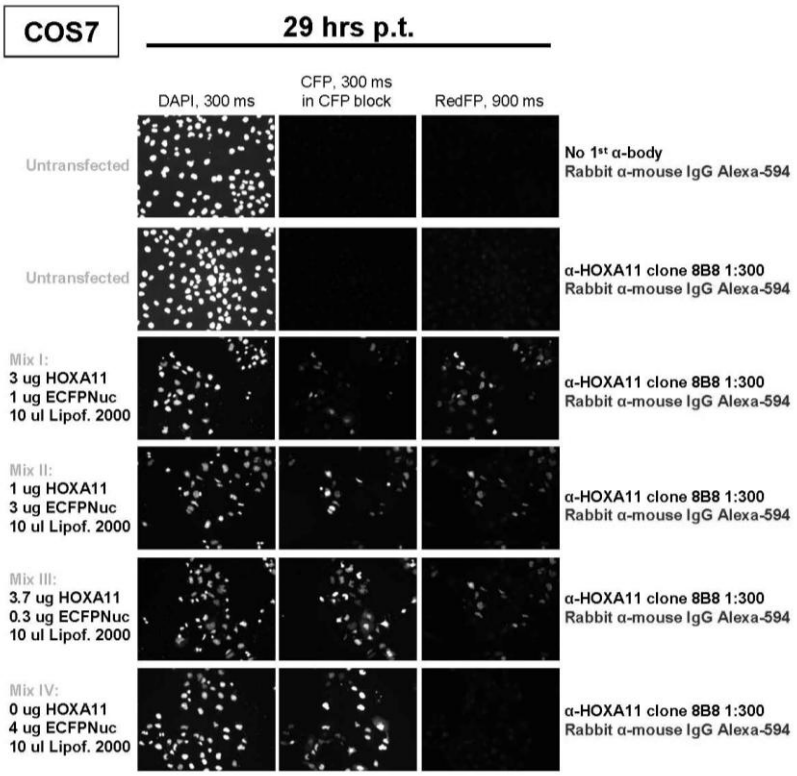
SUPPLEMENTARY TABLES (CHAPTER 6)

Supplementary Table 1 Mouse and human *HOXA* gene-specific primers used in this study for real-time PCR

Gene	**Forward primer (5' - 3')**	**Reverse primer (5' - 3')**
Hoxa1	GGCAGCGATGAGAAAACGGA	GTGGGAGGTAGTCAGAGTGTC
Hoxa2	GTGCAAGGAGAACCAAAACAGC	TGAAAAGTGTACCCTTCCCTCT
Hoxa3	ATTCGCTGGTCAACAGTGTCC	CTGTGTAGCGTTTCTGTGGG
Hoxa4	CGGTGGTGTACCCCTGGAT	GCTTAGGTTCGCCTCCGTTAT
Hoxa5	GCTGCACATTAGTCACGACAA	GCAAAGGGCATGAGCTATTTCG
Hoxa6	ACCTGCACTTTTCTCCCGAG	GGTAAACAGGGCTTGTGTACTTC
Hoxa7	CAGCCCCCTCTATCAGAGC	ATGCGGAAACTGGCTTCGG
Hoxa9	CCACGCTTGACACTCACACTT	CGGCATTGTTTTCGGAGAAGG
Hoxa10	CACCACCCACTCTGGTTTG	TGCATTTTCGCCTTTGGAACT
Hoxa11	TGCGCGAAGTGACCTTCAG	TGGGGTGGTGGTAGACGTT
Hoxa13	ACGGCCAAATGTACTGCCC	TCCCGTTCGAGTTCTTTCAAC
Gene	**Forward primer (5' - 3')**	**Reverse primer (5' - 3')**
HOXA1	CAGATTGGGTGCCAGCATAC	GGAAGCCGGGTTTCTGAAGT
HOXA2	CTTAGTTATTGACCTGGAGACTGG	GTGCACAGGTTCAAGCCATAG
*HOXA3**	CACAGTGGCCAAACAAATCTTCC	CTGATCCTTTTTGTACTCATGCGG
HOXA4	AGCAGGTCTTGGAGCTGGAG	CACACCTGGCAGCCTTGTT
HOXA6	GGCAAAGGCGGGCGAGTAG	CGAGGTCGGGGAGCTCAG
HOXA7	CGCTGCATGGCGCGGCTGAG	GCAGCAGTGGCGGCGGCAGA
HOXA9#	CCCAGCAGCCAACTGGATTACTGCGC	CACTCGTCTTTTGCTCGGTCTTTG
HOXA10	CTGATGAATCTCCAGGCGACG	TGACACTTAGGACAATATCTATCTC
HOXA11	CATTGGATTCTGAGAGCTGTGC	AGTCCACTCTGTGTCGAGGCTT

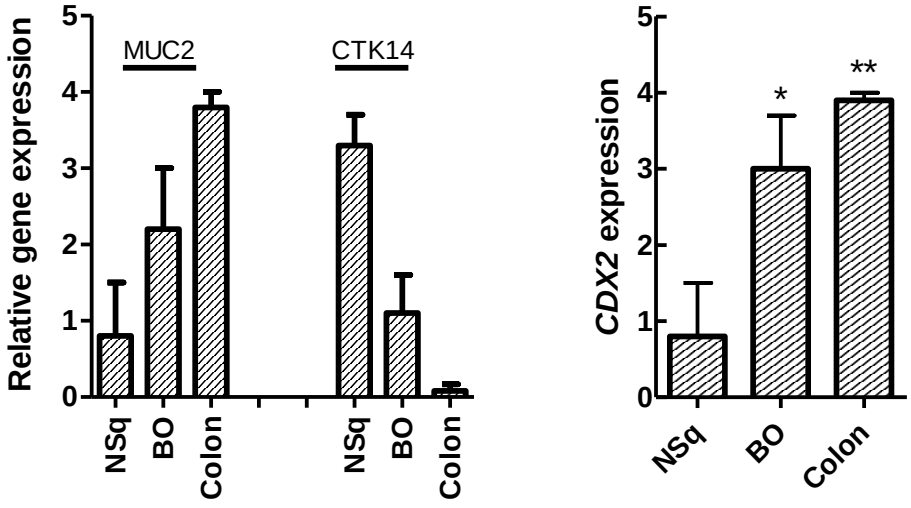
* reference [34]; # reference [35]

SUPPLEMENTARY FIGURES (CHAPTER 6)



Supplementary Figure 1. Transfection
Transfections were performed using basic Fibroblasts Nucleofector® Kit (Amaxa, VPI-1002) and Cell Line Nucleofecter buffer V (Amaxa, VCA1003). COS7 cells were grown on cover slips treated with FNC coating mix (10P's Axxora). Transfection efficiency of approximately 85% were reached. 29 hours after transfection cells were fixed and stained with HOXA11 antibody (clone 8B8; 1:300) and visualised using Alexa-594 fluorescent dye.

Supplementary Figure 2.



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SUPPLEMENTARY METHODS (CHAPTER 7)

CASE PHENOTYPING

No cases were included where doubt may have existed about the phenotype, especially short segments less than 1cm in length or cases where pathology was not consisted with columnar lined metaplasia. All cases conformed to the British Society of Gastroenterology standards for histological criteria of Barrett's. These are the accepted North European criteria as well as in other parts of the world (90.3% had intestinal metaplasia) [1] and [2]. In total 52.7% also had endoscopic segments greater than 3cm in length according the Prague C and M criteria, which is an accepted worldwide objective grading system [2].

DNA SAMPLE PREPARATION

Genomic DNA for all discovery cases was shipped to the Wellcome Trust Sanger Institute (WTSI), Cambridge. Where there was sufficient DNA, quality was validated using the Sequenom iPLEX assay designed to genotype 4 gender SNPs and 26 SNPs present on the Illumina Beadchips. DNA concentrations were quantified using a PicoGreen assay (Invitrogen) and an aliquot assayed by agarose gel electrophoresis. A DNA sample was considered to pass quality control if the DNA concentration was greater than or equal to 50 ng/μl, the DNA was not degraded, the gender assignment from the iPLEX assay matched that provided in the patient data manifest and genotypes were obtained for at least two thirds of the SNPs on the iPLEX.

GWA GENOTYPING

Samples from the case collection were genotyped at the WTSI on the Human660W-Quad (a custom chip designed by WTCCC2 and comprising Human550 and a set of circa 6000 common CNVs from the Structural Variation Consortium [3]) and samples from the UK control collections were genotyped on the Human1.2M-Duo (a WTCCC2 custom array comprising Human1M-Duo and the common CNV content described above). Bead intensity data was processed and normalized for each sample in BeadStudio; data for successfully genotyped samples was extracted and genotypes called within collections using Illuminus [4].

QUALITY CONTROL

Samples: As previously described [5,6], we identified and removed samples whose genome-wide patterns of diversity differed from those of the collection at large, interpreting them as likely to be due to biases or artefacts. To do so we used a Bayesian clustering approach to infer outlying individuals on the basis of call rate, heterozygosity, ancestry and average probe intensity. To obtain a set of putatively unrelated individuals we used a hidden Markov model (HMM) to infer identity by descent along the genome and then iteratively removed individuals to obtain a set with pair-wise identity by descent less than 5%. To guard against sample mishandling we removed samples if their inferred gender was discordant with the

recorded gender or if less than 90% of the SNPs typed by Sequenom on entry to the sample handling (see above) agreed with the genome-wide data. Following sample quality control our final discovery dataset consisted of 1,853 cases and 5,175 controls (Supplementary Table 10).

SNPs: A measure of (Fisher) information for the allele frequency at each SNP was calculated using SNPTEST (http://www.stats.ox.ac.uk/~marchini/software/gwas/snptest.html). Autosomal SNPs were excluded if this information measure was below 0.98, if the minor allele frequency was less than 0.01%, if the SNP had more than 5% missing data, or if the Hardy Weinberg p-value was lower than 10^{-20}. In the 58C, UKBS and case data set, association between the SNP and the plate on which samples were genotyped was calculated and SNPs with a plate effect p-value less than 10^{-6} were also excluded. An additional 45 SNPs were removed following visual inspection of cluster plots. Only SNPs genotyped on all the case and control collections were considered, leaving 521,744 autosomal SNPs after quality control.

STATISTICAL ANALYSIS

GWAS

We performed single SNP analysis in the discovery data sets under an additive model (on the log-odds scale) using missing data likelihood score tests as implemented in SNPTEST. Association analysis was performed in the UK and Dutch replication cohorts separately via a logistic regression assuming an additive genetic model. We then conducted a fixed effect meta-analysis in R to combine the evidence of association across the discovery and replication data.

TESTING FOR INTERACTIONS.

To look for interactions between associated loci, we considered all pairs of index SNPs listed in Supplementary Table 5 and for each pair, we compared two logistic regression models. In the first (null) model, a separate parameter for each SNP specified the multiplicative increase in the odds of disease with each additional copy of the risk allele for that SNP. We compared this, using a likelihood ratio test, to a model for interaction in which there is a third parameter which modifies the effect associated with carrying risk alleles at both SNPs. There are many different ways to model both the marginal effects at each SNP and their joint effect [7], and so there are many approaches to search for interactions. The procedure we adopted is perhaps the simplest (multiplicative model marginally at each SNP and a single additional parameter for multiplicative interactions).

SUPPLEMENTARY TABLES (CHAPTER 7)

Supplementary Table 1. Discovery sample collection cohorts

Site	Number of cases (post QC)
Aberdeen Royal Infirmary, Aberdeen	5 (5)
Addenbrookes Hospital, Cambridge	3 (3)
Alexandra Hospital, Redditch	21 (21)
Altnagelvin Hospital, Londonderry	24 (24)
Antrim & Whiteabbey United Hospitals, Antrim	59 (59)
Ayr Hospital, Ayr	8 (6)
Barnsley Hospital NHS Foundation Trust, Barnsley	23 (23)
Barts and The London, London	23 (20)
Belfast City Hospital, Belfast	13 (13)
Blackpool Victoria Hospital, Blackpool	26 (25)
Bronglais General Hospital, Aberystwyth	19 (17)
City Hospital Birmingham, Birmingham	13 (11)
Conquest Hospital, St Leonard on Sea	7 (7)
Countess of Chester Hospital, Chester	44 (42)
Craigavon Area Hospital, Craigavon	43 (40)
Cumberland Infirmary, Carlisle	26 (24)
Darent Valley Hospital, Dartford	15 (15)
Derriford Hospital, Plymouth	9 (9)
Gartnavel General Hospital, Glasgow	7 (6)
George Eliot Hospital, Nuneaton	8 (8)
Glasgow Royal Infirmary, Glasgow	14 (13)
Gloucestershire Royal Hospital, Gloucester	166 (149)
Good Hope Hospital, Sutton Coldfield	8 (8)
Guy's Hospital, London	12 (10)
Hairmyres Hospital, East Kilbride	12 (12)
Harrogate District Hospital, Harrogate	24 (24)
Hereford County Hospital, Hereford	3 (3)
John Radcliffe Hospital, Oxford	19 (17)
Kettering General Hospital, Kettering	18 (18)
Lagan Valley Hospital, Lisburn	19 (17)

Site	Number of cases (post QC)
Leicester General Hospital, Leicester	111 (96)
Leicester Royal Infirmary, Leicester	137 (131)
Lister Hospital, Stevenage	16 (14)
Maidstone Hospital Kent Oncology Centre, Maidstone	8 (7)
Mater Infirmorium Hospital, Belfast	29 (28)
May Day University Hospital, Croydon	18 (15)
Neath Port Talbot Hospital, Port Talbot	4 (4)
New Cross Hospital, Wolverhampton	7 (7)
Ninewells Hospital, Dundee	41 (40)
Nobles Hospital, Braddan	6 (5)
North Manchester General Hospital, Manchester	18 (17)
North Tyneside General Hospital, North Shields	30 (27)
Northampton General Hospital, Northampton	5 (5)
QEII East & North Hertfordshire NHS Trust, Welwyn Garden City	46 (44)
Queen Alexandra Hospital, Portsmouth	96 (92)
Queen Elizabeth Hospital, Birmingham	4 (4)
Queen Elizabeth Hospital, Kings Lynn	11 (11)
Queen Margaret Hospital , Dunfermline	19 (19)
Queen Mary's Hospital NHS Trust, Sidcup	10 (8)
Queens Medical Centre, Nottingham	52 (48)
Rotherham General Hospital, Rotherham	5 (5)
Royal Albert Edward Infirmary NHS Trust, Wigan	53 (48)
Royal Alexandra Hospital, Paisley	21 (20)
Royal Bournemouth Hospital, Bournemouth	6 (6)
Royal Cornwall Hospital, Truro	8 (8)
Royal Derby Hospital, Derby	35 (31)
Royal Devon & Exeter NHS Foundation Trust, Exeter	2 (2)
Royal Infirmary of Edinburgh, Edinburgh	19 (17)
Royal Lancaster Infirmary, Lancaster	11 (9)
Royal Liverpool Hospital, Liverpool	28 (24)
Royal Oldham Hospital, Oldham	9 (8)
Royal Sussex County Hospital, Brighton	18 (16)
Royal Victoria Hospital, Belfast	56 (56)
Russell's Hall Hospital, Dudley	19 (18)

Site	Number of cases (post QC)
Sandwell General Hospital, Lyndon	24 (18)
Southern General, Glasgow	3 (2)
St Mark's Hospital, Harrow	13 (13)
St Mary's Hospital, London	1 (1)
St Richard's Hospital, Chichester	9 (8)
Stepping Hill Hospital, Stockport	16 (13)
Tameside General Hospital, Ashton-under-Lyne	1 (1)
Taunton & Somerset NHS Trust (Musgrove Park), Taunton	24 (21)
Torbay Hospital, Torquay	55 (49)
Ulster Community Hospitals Trust, Belfast	14 (14)
University Hospitals Coventry and Warwickshire, Coventry	11 (10)
Victoria Hospital, Kirkcaldy	23 (21)
Victoria Infirmary, Glasgow	2 (2)
Wansbeck General Hospital, Ashington	32 (29)
Warwick Hospital, Warwick	12 (9)
Western General Hospital, Edinburgh	74 (70)
Weston General Hopsital , Weston-Super-Mare	2 (2)
Wexham Park Hospital, Slough	8 (8)
Worcestershire Royal Hospital, Worcester	4 (4)
Wycombe Hospital, High Wycombe	4 (4)
Yeovil Hospital, Yeovil	10 (10)
York District Hospital, York	7 (5)

Supplementary Table 2. The top 100 ranked SNPs at which replication was attempted in the UK replication cohort. Separate .xls table

Supplementary Table 3. UK Replication sample collection cohorts

Site	Number of cases (Post QC)
Aberdeen Royal Infirmary, Aberdeen	2 (2)
Addenbrookes Hospital, Cambridge	2 (2)
Alexandra Hospital, Redditch	2 (2)
Antrim & Whiteabbey United Hospitals, Antrim	4 (4)

Site	Number of cases (Post QC)
Barnsley Hospital NHS Foundation Trust, Barnsley	5 (4)
Belfast City Hospital, Belfast	1 (1)
Bishop Auckland Hospital, County Durham	4 (4)
Blackpool Victoria Hospital, Blackpool	31 (22)
Bradford Royal Infirmary, Bradford	7 (7)
Buckinghamshire Hospitals NHS Trust (Stoke Mandeville)	27 (26)
Cheltenham General Hospital, Cheltenham	4 (4)
Chester Le Street Hospital, County Durham	2 (2)
Chorley and South Ribble Hospital, Chorley	4 (3)
Countess of Chester Hospital, Chester	10 (6)
Craigavon Area Hospital, Craigavon	2 (1)
Cumberland Infirmary, Carlisle	6 (6)
Darent Valley Hospital, Dartford	4 (4)
East & North Hertforshire NHS Trust, Welwyn Garden City	17 (17)
Falkirk Royal Infirmary, Falkirk	5 (4)
Gartnavel General Hospital, Glasgow	3 (3)
Gloucestershire Royal Hospital, Gloucester	102 (89)
Great Western Hospital, Swindon	12 (10)
Hairmyres Hospital, East Kilbride	1 (1)
Harrogate District Hospital, Harrogate	21 (17)
Hereford County Hospital, Hereford	1 (1)
Lagan Valley Hospital, Lisburn	5 (2)
Leicester General Hospital, Leicester	41 (35)
Leicester Royal Infirmary, Leicester	224 (186)
Lister Hospital, Stevenage	16 (16)
Luton & Dunstable Hospital, Luton	8 (8)
Maidstone Hospital, Maidstone	9 (8)
Mater Infirmorium Hospital, Belfast	1 (1)
May Day University Hospital, Croydon	1 (1)
Newcross Hospital, Wolverhampton	4 (3)
Ninewells Hospital, Dundee	3 (3)
Nobles Hospital, Braddan	1 (1)
North Devon District Hospital, North Devon	60 (46)
North Tyneside General Hospital, North Shields	7 (1)

Site	Number of cases (Post QC)
Northampton General Hospital, Northampton	2 (1)
QEII East & North Hertfordshire NHS Trust, Welwyn Garden City	6 (5)
Queen Alexandra Hospital, Portsmouth	32 (23)
Queen Elizabeth Hospital, Birmingham	84 (69)
Queen Elizabeth Hospital, Kings Lynn	1 (1)
Queen Margaret Hospital Dunfermline, Dunfermline	24 (17)
Queens Hospital, Burton Hospitals NHS Foundation Trust	3 (3)
Queens Medical Centre, Nottingham	10 (8)
Royal Albert Edward Infirmary NHS Trust, Wigan	29 (26)
Royal Alexandra Hospital, Paisley	2 (1)
Royal Cornwall Hospital, Truro	31 (28)
Royal Devon & Exeter NHS Foundation Trust, Exeter	44 (38)
Royal Infirmary of Edinburgh, Edinburgh	1 (1)
Royal Lancaster Infirmary, Lancaster	1 (1)
Royal Shrewsbury Hospital, Shrewsbury	37 (36)
Royal Sussex County Hospital, Brighton	1 (1)
Royal Victoria Hospital, Belfast	5 (4)
Sandwell General Hospital Lyndon	31 (24)
Shotley Bridge Hospital, County Durham	8 (7)
South Tyneside District Hospital, Southshields	28 (27)
St Mark's Hospital, Harrow	12 (11)
St Richard's Hospital, Chichester	10 (4)
Stafford Hospital, Stafford	10 (10)
Stepping Hill Hospital, Stockport	2 (2)
Stirling Royal Infirmary, Stirling	92 (77)
Taunton & Somerset NHS Trust (Musgrove Park), Taunton	2 (2)
Torbay Hospital, Torquay	48 (45)
Ulster Community Hospitals Trust, Belfast	1 (1)
University Hospital Coventry, Coventry	25 (23)
University Hospital of North Durham, County Durham	3 (3)
University Hospital of North Staffordshire, Staffordshire	11 (7)
University Hospitals Coventry and Warwickshire, Coventry	5 (5)
Victoria Hospital, Kirkcaldy	12 (9)
Wansbeck General Hospital, Ashington	28 (26)

Site	Number of cases (Post QC)
Western General Hospital, Edinburgh	4 (1)
Weston General Hospital, Weston Super Mare	4 (2)
Wexham Park Hospital, Slough	1 (1)
Wycombe General Hospital, High Wycombe	5 (4)
Yeovil Hospital, Yeovil	1 (1)

Supplementary Table 4. Dutch Replication sample collection cohorts

Site	Number of cases (Post QC)
Rotterdam	243 (225)
Nijmegen	115 (115)
Utrecht	75 (75)
Amsterdam	63 (63)

Supplementary Table 5. All loci at which replication was attempted in the UK and Dutch replication populations

Chr	rsID	Position	Risk allele	Cases RAF	Controls RAF	Discovery P value	OR	95% CI	UK rep P value	Dutch P value	Meta P
1	rs7513382	158638733	C	0.496	0.463	1.38×10^{-4}	1.16	1.07-1.25	0.1105	(-) 9.66×10^{-3}	6.61×10^{-3}
2	rs3072	20741887	G	0.408	0.363	2.67×10^{-7}	1.23	1.14-1.33	0.241	0.0868	6.36×10^{-7}
5	rs2731672	176775080	A	0.274	0.240	1.52×10^{-4}	1.18	1.08-1.29	5.02×10^{-3}	0.740	6.57×10^{-6}
6	rs13211507	28365356	A	0.906	0.880	7.64×10^{-6}	1.32	1.17-1.49	0.0349	7.11×10^{-4}	1.43×10^{-8}
6	rs9262143	30760760	G	0.882	0.852	4.94×10^{-7}	1.33	1.19-1.48)	0.0949	0.0214	6.61×10^{-8}
7	rs1860218	153276785	G	0.338	0.306	4.52×10^{-5}	1.19	1.09-1.29	0.163	(-) 0.223	1.02×10^{-3}
7	rs3734960	154315086	G	0.292	0.259	1.89×10^{-5}	1.21	1.11-1.32	0.0398	0.484	5.70×10^{-6}
8	rs13273672	11649790	G	0.344	0.301	8.83×10^{-8}	1.25	1.15-1.36	0.374	(-) 0.230	9.88×10^{-5}
8	rs8180912	11677400	A	0.224	0.191	3.66×10^{-6}	1.25	1.14-1.37	0.151	(-) 0.496	9.22×10^{-5}
13	rs3923500	85657509	G	0.818	0.785	1.69×10^{-5}	1.23	1.12-1.35	0.0509	0.0237	4.78×10^{-7}
15	rs2218260	56001502	G	0.433	0.393	4.94×10^{-5}	1.17	1.09-1.27	0.0492	0.297	8.45×10^{-6}
15	rs2535483	95357916	A	0.273	0.238	3.41×10^{-5}	1.20	1.10-1.31	0.0853	0.0134	1.02×10^{-6}
16	rs2043633	5759275	A	0.629	0.588	6.21×10^{-5}	1.17	1.08-1.27	0.0198	0.0394	5.86×10^{-7}
19	rs10423674	18678903	G	0.700	0.661	4.87×10^{-5}	1.18	1.09-1.28	0.412	0.443	2.53×10^{-4}

(-) Direction of risk opposite to that in the discovery population. All P values are 2-sided.

Supplementary Table 6. Table of association with imputed classical HLA alleles. HLA alleles imputed using HLA*IMP

	Discovery						UK replication						Meta		
HLA allele	**Cases Allele Freq**	**Con Allele Freq**	**missing data proportion**	**Beta**	**SE**	**P value**	**cases maf**	**controls maf**	**missing data proportion**	**Beta**	**SE**	**P value**	**Beta**	**SE**	**P value**
HLA-C*701	**0.15**	**0.18**	**0.02**	**-0.17**	**0.05**	**9.63E-04**	**0.17**	**0.18**	**0.05**	**-0.01**	**0.06**	**0.87**	**-0.10**	**0.04**	**8.23E-03**
HLA-DQB*602	0.16	0.14	0.02	0.18	0.05	1.08E-03	0.13	0.14	0.10	-0.02	0.07	0.82	0.11	0.04	1.44E-02
HLA-DQB*201	0.13	0.15	0.02	-0.17	0.06	1.87E-03	0.17	0.16	0.10	0.04	0.07	0.59	-0.09	0.04	4.32E-02
HLA-B*801	**0.13**	**0.15**	**0.05**	**-0.17**	**0.06**	**2.18E-03**	**0.13**	**0.14**	**0.03**	**-0.10**	**0.07**	**0.13**	**-0.14**	**0.04**	**9.12E-04**
HLA-DQA*501	0.22	0.24	0.05	-0.12	0.05	7.82E-03	0.22	0.24	0.06	-0.11	0.06	0.05	-0.12	0.04	9.38E-04
HLA-DQA*102	0.20	0.18	0.05	0.12	0.05	1.43E-02	0.19	0.18	0.06	0.04	0.06	0.56	0.09	0.04	2.38E-02
HLA-A*101	**0.18**	**0.20**	**0.03**	**-0.12**	**0.05**	**1.79E-02**	**0.18**	**0.19**	**0.06**	**-0.07**	**0.06**	**0.27**	**-0.10**	**0.04**	**1.15E-02**
HLA-DQB*302	0.09	0.10	0.02	-0.13	0.06	3.56E-02	0.11	0.10	0.10	0.08	0.08	0.33	-0.05	0.05	2.95E-01
HLA-A*2501	0.03	0.02	0.03	0.26	0.13	4.79E-02	0.02	0.02	0.06	0.21	0.19	0.27	0.24	0.11	2.40E-02

Supplementary Table 7. Results for SNPs associated with Circumference (C) and Maximal length (M) measurement of the Barrett's segment at $P<10^{-5}$.

SNPs associated with C measurements

						DISCOVERY		
CHR	**MARKER**	**POSITION**	**A1**	**A2**	**MAF**	**BETA**	**SE**	**P**
3	rs1912785	147884861	A	G	0.41	-0.15	0.03	$7.81x10^{-6}$
12	rs1531228	13520648	A	G	0.14	0.20	0.05	$9.74x10^{-6}$
15	rs12901001	84660480	A	G	0.35	0.15	0.03	$5.38x10^{-6}$

SNPs associated with M measurements

						DISCOVERY			UK REPLICATION			DUTCH REPLICATION			META		
CHR	**MARKER**	**POSITION**	**A1**	**A2**	**MAF**	**BETA**	**SE**	**P**	**BETA**	**SE**	**P**	**BETA**	**SE**	**P**	**BETA**	**SE**	**P**
9	rs10123313	4278098	A	G	0.01	-0.72	0.14	$1.61x10^{-7}$	-0.12	0.17	0.48	0.15	0.39	0.70	-0.44	0.10	$1.87x10^{-5}$
5	rs821735	111695471	A	C	0.11	-0.23	0.05	$7.78x10^{-6}$									
5	rs1004385	111706536	A	G	0.11	0.25	0.05	$1.23x10^{-6}$									
5	rs6594591	111719360	A	G	0.10	-0.24	0.05	$6.21x10^{-6}$									
12	rs12425938	60770659	A	G	0.15	-0.19	0.04	$9.06x10^{-6}$									
12	rs9669389	60776776	A	G	0.15	-0.19	0.04	$9.41x10^{-6}$									

Supplementary Table 8. Sex-stratified analysis of 16 SNPs for which replication was attempted. Male-only analysis (M): Discovery: 1488 cases, 2611 controls. UK replication: 785 cases, 3270 controls. NL replication:354 cases, 660 controls. Female-only analysis (F): Discovery 365 cases, 2564 controls. UK replication: 320 cases, 3512 controls. NL replication: 124 cases, 487 controls.

			Discovery (Dis)		UK Replication		NL Replication		Meta		$P_{interaction}$				$P_{Gender-Heterogeneity}$
SNP	Sex	Risk allele	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	Dis	UK rep	NL rep	meta	
rs7513382	M	C	1.16 (1.06-1.28)	$1.18x10^{-3}$	1.04 (0.93-1.17)	0.44	0.78 (0.65-0.93)	$6.06x10^{-3}$	1.06 (0.99-1.13)	0.08	0.46	0.4	0.28	0.13	0.2
	F	C	1.24 (1.06-1.26)	$6.15x10^{-3}$	1.14 (0.97-1.33)	$6.29x10^{-3}$	0.93 (0.71-1.23)	0.62	1.15 (1.04-1.27)	$8.07x10^{-3}$					
rs3072	M	G	1.23 (1.12-1.36)	$1.16x10^{-5}$	1.03 (0.92-1.16)	0.56	1.2 (0.99-1.45)	0.06	1.15 (1.08-1.23)	$3.01x10^{-5}$	0.53	0.85	0.47	0.55	0.45
	F	G	1.16 (0.98-1.35)	0.08	1.05 (0.89-1.25)	0.08	1.06 (0.80-1.40)	0.69	1.1 (0.99-1.22)	0.09					
rs2731672	M	A	1.1 (1.00-1.23)	0.06	1.12 (0.99-1.28)	0.07	1.02 (0.82-1.26)	0.88	1.1 (1.02-1.19)	0.01	0.03	0.4	0.47	0.01	0.02
	F	A	1.4 (1.40-1.67)	$2.08x10^{-4}$	1.24 (1.03-1.50)	$2.05x10^{-4}$	1.17 (0.86-1.58)	0.32	1.3 (1.15-1.46)	$1.65x10^{-5}$					
rs13211507	M	A	1.42 (1.23-1.64)	$2.23x10^{-6}$	1.25 (1.05-1.50)	0.01	1.45 (1.08-1.94)	0.01	1.36 (1.23-1.51)	$7.58x10^{-9}$	0.14	0.13	0.65	0.04	0.06
	F	A	1.14 (0.63-1.46)	0.3	1 (0.78-1.29)	0.3	1.59 (1.03-2.46)	0.04	1.13 (0.96-1.33)	0.01					
rs9257809	M	A	1.47 (1.28-1.69)	$7.10x10^{-8}$	1.23 (1.04-1.46)	0.02	1.48 (1.12-1.95)	$6.45x10^{-3}$	1.38 (1.25-1.53)	$3.41x10^{-10}$	0.1	0.11	0.84	0.01	0.02
	F	A	1.16 (0.73-1.47)	0.23	1.03 (1.31-.80)	0.24	1.39 (0.90-2.16)	0.14	1.1 (0.94-1.29)	0.23					
rs9262143	M	A	1.39 (1.22-1.58)	$9.63x10^{-7}$	1.2 (1.02-1.41)	0.02	1.32 (1.01-1.73)	0.04	1.31 (1.19-1.44)	$2.14x10^{-8}$	0.51	0.09	0.78	0.06	0.06
	F	G	1.24 (0.99-1.40)	0.06	1.05 (0.84-1.32)	0.06	1.23 (0.81-1.86)	0.32	1.1 (0.95-1.28)	0.19					
rs1860218	M	G	1.18 (1.07-1.31)	$8.91x10^{-4}$	1.07 (0.95-1.21)	0.26	0.9 (0.74-1.09)	0.28	1.1 (1.03-1.18)	$8.05x10^{-3}$	0.82	0.94	1	0.75	0.96
	F	G	1.21 (1.02-1.28)	0.03	1.08 (0.91-1.29)	0.03	0.9 (0.67-1.21)	0.47	1.1 (0.99-1.24)	0.08					
rs3734960	M	G	1.18 (1.06-1.31)	$1.74x10^{-3}$	1.08 (0.96-1.22)	0.2	1.08 (0.87-1.34)	0.48	1.13 (1.05-1.22)	$1.10x10^{-3}$	0.87	0.9	0.85	0.95	0.99
	F	G	1.2 (1.00-1.32)	0.05	1.1 (0.91-1.32)	0.05	1.04 (0.76-1.43)	0.81	1.13 (1.01-1.28)	0.04					

SNP	Sex	Risk allele	Discovery (Dis) OR (95%CI)	P	UK Replication OR (95%CI)	P	NL Replication OR (95%CI)	P	Meta OR (95%CI)	P	$P_{interaction}$ Dis	UK rep	NL rep	meta	$P_{Gender-Heterogeneity}$
rs13273672	M	G	1.23 (1.12-1.36)	$2.26x10^{-5}$	1 (0.89-1.13)	0.93	1 (0.82-1.21)	0.98	1.12 (1.04-1.20)	$2.01x10^{-3}$	0.87	0.54	0.05	0.53	0.48
	F	G	1.21 (1.02-1.28)	0.03	1.07 (0.90-1.28)	0.02	0.71 (0.53-.95)	0.02	1.06 (0.95-1.19)	0.28					
rs8180912	M	A	1.21 (1.08-1.35)	$7.57x10^{-4}$	1.08 (0.94-1.24)	0.27	1.02 (0.82-1.28)	0.86	1.14 (1.05-1.23)	$1.67x10^{-3}$	0.7	0.67	0.16	0.52	0.49
	F	A	1.27 (1.23-1.55)	0.02	1.02 (0.83-1.26)	0.02	0.77 (0.55-1.08)	0.13	1.08 (0.95-1.23)	0.26					
rs3923500	M	G	1.18 (1.05-1.33)	$4.39x10^{-3}$	1.11 (0.97-1.27)	0.14	1.19 (0.95-1.49)	0.14	1.15 (1.06-1.25)	$5.39x10^{-4}$	0.31	0.73	0.37	0.28	0.28
	F	G	1.31 (1.08-1.32)	$5.41x10^{-3}$	1.15 (0.95-1.40)	$5.34x10^{-3}$	1.43 (1.00-2.05)	0.05	1.25 (1.10-1.43)	$4.99x10^{-4}$					
rs2218260	M	G	1.19 (1.09-1.31)	$2.24x10^{-4}$	1.13 (1.01-1.26)	0.04	1.07 (0.89-1.29)	0.48	1.15 (1.08-1.23)	$3.62x10^{-5}$	0.36	0.33	0.94	0.14	0.18
	F	G	1.1 (0.94-1.80)	0.25	1.02 (0.86-1.20)	0.25	1.06 (0.80-1.40)	0.71	1.06 (0.95-1.17)	0.3					
rs2535483	M	A	1.17 (1.05-1.30)	$2.89x10^{-3}$	1.12 (0.98-1.27)	0.09	1.14 (0.93-1.41)	0.21	1.15 (1.07-1.24)	$2.93x10^{-4}$	0.24	0.38	0.27	0.47	0.52
	F	A	1.34 (1.33-1.60)	$1.64x10^{-3}$	1 (0.83-1.22)	$1.66x10^{-3}$	1.43 (1.04-1.98)	0.03	1.21 (1.07-1.36)	$2.83x10^{-3}$					
rs2043633	M	A	1.11 (1.02-1.22)	0.02	1.12 (1.00-1.26)	0.04	1.18 (0.98-1.42)	0.07	1.13 (1.05-1.20)	$4.43x10^{-4}$	0.03	0.64	0.71	0.46	0.34
	F	A	1.36 (1.36-1.60)	$1.63x10^{-4}$	1.07 (0.91-1.26)	$1.67x10^{-4}$	1.11 (0.83-1.47)	0.48	1.2 (1.08-1.33)	$9.44x10^{-4}$					
rs9936833	M	G	1.19 (1.09-1.31)	$2.34x10^{-4}$	1.18 (1.05-1.32)	$4.06x10^{-3}$	1.15 (0.95-1.40)	0.15	1.18 (1.11-1.27)	$1.10x10^{-6}$	0.65	0.62	0.11	0.86	0.98
	F	G	1.24 (1.06-1.26)	$8.28x10^{-3}$	1.24 (1.05-1.47)	$8.15x10^{-3}$	0.86 (0.63-1.16)	0.32	1.19 (1.06-1.32)	$2.15x10^{-3}$					
rs10423674	M	C	1.15 (1.04-1.27)	$5.36x10^{-3}$	1 (0.89-1.13)	0.93	1 (0.82-1.22)	0.98	1.08 (1.00-1.15)	0.04	0.53	0.25	0.39	0.21	0.15
	F	C	1.23 (1.06-1.26)	0.01	1.14 (0.96-1.35)	0.01	1.17 (0.87-1.58)	0.3	1.18 (1.06-1.32)	$2.74x10^{-3}$					

Supplementary Table 9. Results for SNPs associated with body mass index (BMI) or waist-hip ratio (WHR) that are genotyped in the discovery data. Risk alleles are given for the forward strand.

Chr	SNP	Position	Phenotype	Risk Allele	Gene	Reference	Risk allele discovery	P Discovery
1	rs1514175	74,764,232	BMI	A	*TNNI3K*	[8]	A	0.78
1	rs1011731	170,613,171	WHR	G	*DNM3-PIGC*	[9]	G	0.75
2	rs2867125	612,827	BMI	C	*TMEM18*	[8]	T	0.83
2	rs10195252	165,221,337	WHR	T	*GRB14*	[9]	C	0.54
3	rs6795735	64,680,405	WHR	C	*ADAMTS9*	[9]	C	0.27
3	rs13078807	85,966,840	BMI	G	*CADM2*	[8]	G	0.086
4	rs13107325	103,407,732	BMI	T	*SLC39A8*	[8]	C	0.92
6	rs987237	50,911,009	BMI/WC	G	*TFAP2B*	[10]	G	0.12
7	rs1055144	25,837,634	WHR	T	*NFE2L3*	[9]	T	0.40
9	rs10968576	28,404,339	BMI	G	*LRRN6C*	[8]	G	0.085
12	rs718314	26,344,550	WHR	G	*ITPR2-SSPN*	Heid et al (Nature Genetics 2010)	A	0.74
12	rs7138803	48,533,735	BMI	A	*FAIM2*	[8]	A	0.65
12	rs1443512	52,628,951	WHR	A	*HOXC13*	Heid et al (Nature Genetics 2010)	A	0.078
14	rs10146997	79,014,915	BMI/WC	G	*NRXN3*	[11]	G	0.0090
15	rs2241423	65,873,892	BMI	G	*MAP2K5*	[8]	G	0.14
18	rs571312	55,990,749	BMI	A	*MC4R*	[8]	A	0.095
18	rs12970134	56,035,730	BMI/WC	A	*MC4R*	[12]	A	0.11
19	rs29941	39,001,372	BMI	G	*KCTD15*	[8]	A	0.60
19	rs2287019	50,894,012	BMI	C	*QPCTL*	[8]	C	0.091

Results in the BE discovery scan for tags of SNPs associated with BMI or WHR that were not genotyped in the discovery data. The genotyped tag SNPs are within 250kb and with $r^2 > 0.8$ (according to 1000G CEU pilot1 June release). Risk alleles are given for the forward strand.

Chr	SNP	Pos	Phenotype	Risk Allele	Gene	Citation	Tag SNP (r^2)	Tagged risk allele	Tag SNP P
1	rs2815752	72,585,028	BMI	A	*NEGR1*	[8]	rs3101336 (0.96)	A	0.78
1	rs1555543	96,717,385	BMI	C	*PTBP2*	[8]	rs11165643 (1)	C	0.23
1	rs984222	119,305,366	WHR	G	*TBX15-WARS2*	[9]	rs10923724 (0.85)	G	0.47
1	rs543874	176,156,103	BMI	G	*SEC16B*	[8]	rs10913469 (0.96)	G	0.91
2	rs713586	25,011,512	BMI	C	*RBJ*	[8]	rs6752378 (1)	T	0.44

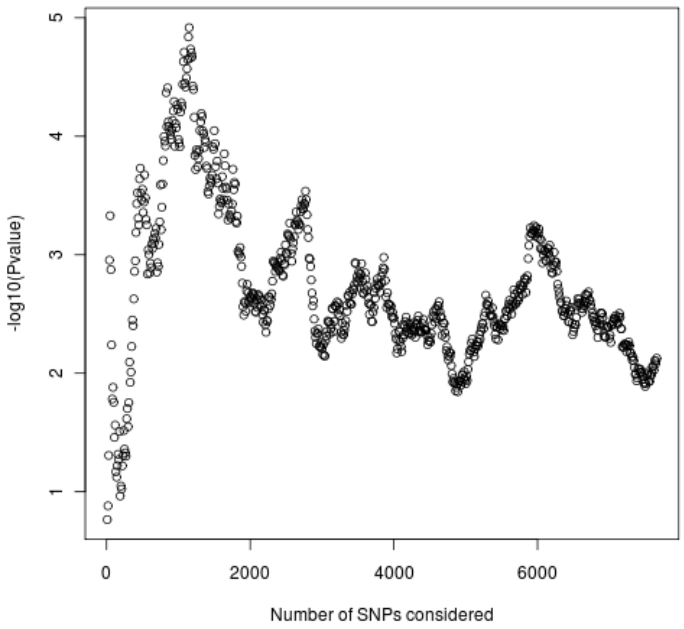
2	rs887912	59,156,381	BMI	T	*FANCL*	[8]	rs759250 (0.92)	T	0.94
4	rs10938397	44,877,284	BMI	G	*GNPDA2*	[8]	rs12641981 (0.90)	A	0.75
5	rs2112347	75,050,998	BMI	T	*FLJ35779*	[8]	rs253414 (0.81)	T	0.51
5	rs4836133	124,360,002	BMI	A	*ZNF608*	[8]	rs6864049 (1)	A	0.085
5	rs6861681	173,295,064	WHR	A	*CPEB4*	[9]	rs7736263 (1)	A	0.37
6	rs1294421	6,688,148	WHR	G	*LY86*	[9]	rs1294433 (0.81)	T	0.98
6	rs206936	34,410,847	BMI	G	*NUDT3*	[8]	rs3798560 (1)	G	0.71
6	rs9491696	127,494,332	WHR	G	*RSPO3*	[9]	rs7766106 (0.96)	G	0.78
11	rs4929949	8,561,169	BMI	C	*RPL27A*	[8]	rs725502 (0.87)	T	0.78
11	rs10767664	27,682,562	BMI	A	*BDNF*	[8]	rs11030104 (0.90)	A	0.031
11	rs3817334	47,607,569	BMI	T	*MTCH2*	[8]	rs7124681 (0.94)	T	0.16
16	rs12444979	19,841,101	BMI	C	*GPRC5B*	[8]	rs12446632 (0.88)	C	0.082
16	rs7359397	28,793,160	BMI	T	*SH2B1*	[8]	rs4788102 (0.96)	C	0.61
16	rs1558902	52,361,075	BMI	A	*FTO*	[8]	rs9930333 (0.90)	A	0.56
19	rs3810291	52,260,843	BMI	A	*TMEM160*	[8]	rs2303108 (1)	G	0.025
22	rs4823006	27,781,671	WHR	A	*ZNRF3-KREMEN1*	[9]	rs2294239 (0.90)	A	0.23

Supplementary Table 10. Sample exclusions in the discovery data

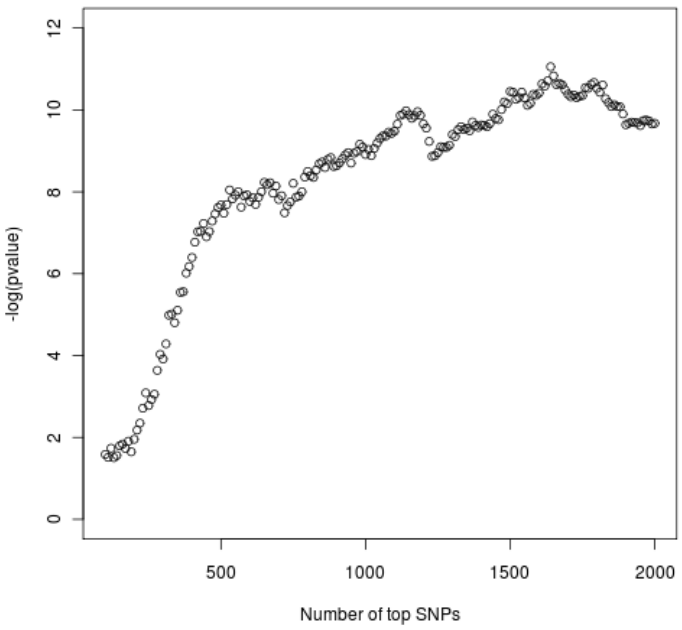
Numbers of individuals excluded where column headings are: PreQC: the number of samples for which genotypes were available, Intensity: signal intensity from the chip outlying, Gender: gender modeled from X chromosome intensity mismatched with supplier gender, Het/CR: call rate and heterozygosity, Ancestry: HapMap PCA population exclusions Identity: <90% concordant with initial Sequenom genotypes, Relatedness: >5%IBD, Phenotype: Incorrect assignment of phenotype. Unique Exc: the number of unique sample excluded, PostQC: the number of samples carried through to the analysis stage.

	PreQC	Intensity	Gender	Het/CR	Ancestry	Identity	Related	Phenotype	Unique Exc	PostQC
BE	1991	1	10	67	28	14	23	2	138	1853
58C	2930	32	11	163	57	1	19	0	256	2674
UKBS	2737	23	14	111	51	8	52	0	236	2501

SUPPLEMENTARY FIGURES (CHAPTER 7)



Supplementary Figure 1. Results of the sign test showing the two-sided P values for the number of pruned SNPs that have the same direction of effects in the discovery and replication samples.



Supplementary Figure 2. Results of the score analysis where a score is calculated for each replication individual, based on the number of risk alleles carried by each individual weighted by the log of the odds ratio from the discovery data. The P value is testing for evidence of higher scores in the cases sample compared to the controls sample.

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APPENDIX

DANKWOORD

CURRICULUM VITAE

PUBLICATIONS

PORTFOLIO

DANKWOORD

De meeste mensen weten dat ik het liefst alles zelf doe. Toch heb ik de afgelopen jaren hulp en steun gekregen van een flink aantal mensen, en dat was nodig, want promoveren kun je echt niet alleen. Onmisbare ingrediënten voor dit boekje waren een goede begeleiding, samenwerking en een flinke scheut sociaal netwerk. De volgende chefkoks, sous-chefs, sommeliers en obers wil ik graag bedanken:

Begeleiding

Om te beginnen zijn dat mijn promotoren. Beste Ernst, jij hebt voor mij meer gedaan dan een promovendus van zijn promotor kan verwachten. Het is bewonderenswaardig dat, hoe druk jouw dagen ook waren, je altijd tijd maakte om een kritische blik op mijn onderzoek te werpen. Die momenten waren enorm leerzaam en heb ik zeer gewaardeerd. Naast je enthousiaste begeleiding heb ik jou ook leren kennen als zeer meelevend en vriendelijk persoon. Ik zal de tijd die ik van jou gekregen heb om bij mijn familie te zijn nooit vergeten.

Later heb ik nog een tweede promotor gekregen. Maikel, jij begon twee jaar geleden aan je positie op het lab en meteen voelde ik me niet meer zo alleen op mijn Barrett-eilandje. Je aanstekelijke energie gaf mij mijn passie terug toen ik die even kwijt was. Ik heb enorm gelachen en geleerd van jouw fascinerende presentaties en (al dan niet wetenschappelijke) anekdotes. Bedankt voor al je interesse en positieve toevoegingen aan mijn onderzoek.

Dan wil ik graag mijn copromotor bedanken. Luc, toen er een begeleider gezocht werd voor mij heb jij die taak dapper op je genomen. Ik ben zeker niet de makkelijkste promovendus geweest, maar jij hebt je altijd vol overgave ingezet om mij over de eindstreep te krijgen. Zonder jou was mij dat ook zeker niet gelukt. Je was er niet alleen met goede ideeën en een frisse blik op het onderzoek, maar zeker ook met veel begrip, geduld en medeleven. Tot op het laatst kon ik onvoorwaardelijk op je rekenen, daarvoor ben ik je erg dankbaar.

Samenwerkingen

Veel artsen, onderzoekers, pathologen en patiënten hebben bijgedragen aan het tot stand komen van de studies in dit boekje. Het begon ooit met Hans en Peter. Peter, mijn eerste gesprek met jouw zal ik nooit vergeten; ik was meteen onder de indruk van je enthousiasme, werklust en stimulerende woorden. Hans, met veel plezier en overgave heb jij je over mij ontfermd in de beginfase van mijn onderzoek. Bedankt dat jullie dit avontuur met mij zijn aangegaan. Aan alle collega's van de Barrett's

groep, inclusief Monique en Min; bedankt voor jullie prettige samenwerking. Een speciaal woord van dank aan Mark van Blankenstein. Met plezier heb ik gebruik kunnen maken van jouw kennis en ervaring wat herhaaldelijk geleid heeft tot unieke en waardevolle benaderingen van mijn onderzoek.

Verder wil ik alle co-auteurs hartelijk bedanken voor hun bijdrage. Ik heb veel medewerking gekregen van de MDL endoscopie unit; Peter Mensink, Manon Spaander, Michel, Diana en Marieke en alle anderen, bedankt voor jullie tomeloze geduld en jullie hulp bij het verzamelen van biopten. Verder dank aan alle patiënten die door hun deelname mij in staat hebben gesteld het translationele onderzoek te doen wat ik zo graag wilde.

Een aanzienlijk deel van een promotieonderzoek is administratie en daarin zijn secretaresses van groot belang. Lieve Linda, Wendy, Leonie en Bernadette, bedankt dat jullie er waren voor administratieve en persoonlijke adviezen.

Collega's

Collega's kwamen er in vele soorten en maten. Zo zijn er de "collega's-van-het-begin": biertjes in Dizzy en op karaoke borrels met Jeroen P, Brenda, Mark, Clara, Alice, Pieter-Jan, Martijn, Angela H, Jeroen F, Paulette en iedereen die ik niet noem! Dan de "collega's-van-daarna": altijd interesse en heel veel lol op onder andere onze kampeertripjes met Antoine, Jasper, Werner, Gertine, Buddy, Angela, Aria, Petra en mijn kamergenoten in het bijzonder Lianne, Suomi, Wendy en Elvira. Jullie zijn alle vier wereldvrouwen! Dan zijn er nog de "ze-zijn-er-nog-steeds-collega's": wat heb ik veel steun en bemoedigende woorden gehad van het eerste Rotterdamse biertje tot het laatste loodje van onder meer Thonie, Patrick, Paula, Jan, Martine, Raymond, en Ozlem. Rest nog de "ex-collega's-die-nog-altijd-bij-je-zijn": Katinka, buiten enthousiasme en onuitputtelijke werklust voegde jij de broodnodige structuur in mijn experimenten toe. Tnx! Marjolein S, we hebben heel wat meegemaakt samen en elkaar vaak door dalen moeten sleuren, maar ik heb er een hele mooie vriendschap aan overgehouden! Marjoleine, chica! Jij bent er echt altijd voor mij, werk of privé, goede en slechte tijden. Ik bewonder je om je sociale gave en inlevingsvermogen. Scot, you have been close to me since day one. We share a passion for science and one for food. We seem to speak the same language, and that is something I share with only few people! I am so happy that you agreed to be my paranimf.

Vrienden

Marta, “Sleeping Beauty” heeft ons bij elkaar gebracht en dat is in enkele jaren uitgegroeid tot een hele speciale vriendschap. Ik kan je alles vertellen, hoewel je meestal weet wat ik denk voordat ik het zelf weet. We hebben al veel gedeeld, verdriet, hilarische momenten, spierpijn, een appartement en heel veel prosecco’s en cocktails. Super dat jij mijn paranimf bent!

Een promovendus heeft ook absoluut steun nodig van mensen die niet aan medische wetenschap verbonden zijn. Met name Nienke en Deem bedankt voor alle gesprekken en lunches die mij eraan herinnerden dat er meer is dan wetenschap! Een speciaal woord voor “Hans van de 12”. Telkens weer toverde jij een heerlijke glimlach op onze gezichten door je positieve uitstraling nog voor wij een wijntje of een La Chouffe hadden besteld. Margriet bedankt voor al onze mooie muzikale momenten die mij positieve energie hebben gegeven. Lieve Isabel, door onze gesprekken heb ik veel over mezelf geleerd en ben ik enorm gegroeid als mens; een kado van onschatbare waarde!

Familie

Ze zeggen weleens dat je vrienden kiest maar niet je familie. Nou, soms voelt het alsof Lech en Ulla mij als lid van hun familie hebben gekozen. Jullie armen stonden altijd voor me open. Iza, je bent meer een vriendin dan een zusje, en ik hoop dat we nog vaak samen biertjes gaan doen! Mijn grote zus Tamara, die samen met haar fantastische gezin mij laat zien wat de wereld te bieden heeft. Supervrouw! De foto’s van Kiki, Mila en Tigo zijn zeker de laatste maanden lichtpuntjes geweest tijdens donkere schrijfnachten.

Lieve Mam, je zei altijd dat je niks van onderzoek weet en mij daar niet mee kon helpen, maar je hebt geen idee! Je hebt me in mijn leven dingen gegeven die onmisbaar waren om te promoveren: doorzettingsvermogen, organisatorisch inzicht, creativiteit en vele wijze levenslessen. Bedankt voor alles. Lieve papa, fijn dat je nog altijd bij me bent, in mijn perfectionisme, in elke vrije trap die perfect “in de kruutsing” draait, in alle flauwe grapjes die ik hoor en in de harten van velen. Fijn dat je tijdens het kaarten op mijn schouder zit en me geluk brengt!

Tot slot mijn lieve Kevin, onwaarschijnlijk hoe jij het de afgelopen maanden met mij hebt volgehouden. Vanaf onze “eerste date” kon ik al rekenen op jouw begrip, steun en geduld. Ik denk dat we na deze zware periode wel wat tijd hebben verdiend om te genieten van de goede dingen van het leven!

CURRICULUM VITAE

Anouk Van de Winkel werd op 13 februari 1983 geboren in Roermond. In 2001 heeft zij haar gymnasiumdiploma met onder meer de vakken biologie en scheikunde behaald aan de Scholengemeenschap St. Ursula te Horn. In hetzelfde jaar is zij aan de Universiteit Maastricht begonnen met de studie Algemene Gezondheidswetenschappen. Tijdens haar studie heeft zij een jaarwerkstuk geschreven met als titel “The role of DNA adducts in the relation between chronic inflammatory disease and carcinogenesis”. Tijdens deze studie heeft zij voorts een stage gevolgd op de afdeling Pathologie van het Academisch Ziekenhuis Maastricht met als thema “Vascularization and immune response in human cutaneous melanomas”. Op 31 oktober 2005 werd de studie Algemene Gezondheidswetenschappen met als afstudeerrichting Biologische Gezondheidskunde succesvol afgerond. Op 1 maart 2006 begon zij aan haar promotieonderzoek naar de etiologie van de Barrett slokdarm op de afdeling Maag-, Darm- en Leverziekten aan het Erasmus MC - Universitair Medisch Centrum te Rotterdam. Zij heeft haar onderzoek verricht onder begeleiding van haar promotoren Prof.dr. E.J. Kuipers en Prof.dr. M.P. Peppelenbosch en copromotor Dr. L.J.W. van der Laan (afdeling Heelkunde).

PUBLICATIONS

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- **van de Winkel A**, Massl R, Ernst J. Kuipers EJ, van der Laan LJW, Peppelenbosch MP. Digestive Disease Week 2011: Highlights of clinical and preclinical research on Barrett's esophagus and associated esophageal adenocarcinoma. Conditionally accepted Diseases of the Esophagus.

- **van de Winkel A**, Menke V, Capello A, Moons LM, Pot RG, van Dekken H, Siersema PD, Kusters JG, van der Laan LJ, Kuipers EJ. Expression, localization and polymorphisms of the nuclear receptor PXR in Barrett's esophagus and esophageal adenocarcinoma. BMC Gastroenterol 2011;11:108.

- **van de Winkel A**, van Zoest KPM, van Dekken H, Moons LMG, Kuipers EJ, van der Laan LJW. Differential expression of the nuclear receptors FXR and PXR for grading dysplasia in patients with Barrett's esophagus. Histopathology 2011;58(2):246-5.

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- Hillen F, Baeten CI, **van de Winkel A**, Creytens D, van der Schaft DW, Winnepenninckx V, Griffioen AW. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. Cancer Immunol Immunother 2008;57(1):97-106.

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PHD PORTFOLIO SUMMARY

Name PhD student:	Anouk Van de Winkel
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PhD period:	2006 - 2011
Promotores:	Prof.dr. E.J. Kuipers
	Prof.dr. M.P. Peppelenbosch
Supervisor:	Dr. L.J.W. van der Laan

General courses	Year
- Biomedical English Writing and Communication	2008
- Genetic polymorphisms	2007
- Molecular Diagnostics	2007

Conferences: oral presentation	Year
- "Vitamin D receptor gene polymorphisms in the 1c promoter region are associated with the risk for erosive esophagitis, Barrett's Esophagus and esophageal cancer". *13th Molecular Medicine day, MolMed Erasmus Postgraduate School*	2009
- "Etiology of Barrett's Esophagus: Bile acid induced expression of HOX genes in the human esophagus". *Tumor celbiologie symposium van KWF Kankerbestrijding*	2008
- "The xenobiotic sensor PXR is associated with Barrett's Esophagus". *Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie*	2007

Conferences: poster presentations	Year
- "The nuclear receptors FXR and PXR as early markers in neoplastic progression in patients with Barrett's Esophagus". *Digestive disease week, Chicago*	2009
- "Vitamin D receptor gene polymorphisms in the 1c promoter region are associated with the risk of esophageal (pre-)malignancies". *Digestive disease week, Chicago*	2009
- "The nuclear receptors FXR and PXR as early marker in neoplastic progression in patients with Barrett's Esophagus". *Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie*	2009
- "Vitamin D receptor gene polymorphisms in the 1c promoter region are associated with the risk of esophageal (pre-)malignancies". *Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie*	2009

Conferences: poster presentations	**Year**
- "Etiology of Barrett's Esophagus: Expression of HOX genes in the human esophagus". *Digestive disease week, San Diego*	2008
- "Etiology of Barrett's Esophagus: Expression of HOX genes in the human esophagus". *Voorjaarsvergadering Nederlandse Vereniging voor Gastroenterologie*	2008
- "Etiology of Barrett's Esophagus: Expression of HOX genes in the human esophagus". *12th Molecular Medicine day, MolMed Erasmus Postgraduate School*	2008
- "The xenobiotic sensor PXR is associated with Barrett's Esophagus". *Digestive disease week, Washington DC*	2007
- "The xenobiotic sensor PXR is associated with Barrett's Esophagus". *11th Molecular Medicine day, MolMed Erasmus Postgraduate School*	2007

International conferences	**Year**
- Digestive disease week, Chicago, 7 May 2011	2011
- Digestive disease week, Chicago, 31 May 2009	2009
- Digestive disease week, San Diego, 19 May 2008	2008
- Digestive disease week, Washington DC, 20 May 2007	2007

Memberships	**Year**
- Dutch Society of Gastroenterology (NVGE)	All
- Section Experimental Gastroenterology (SEG)	All