þÿIntrinsic Cellular Susceptibility to Barrett s Eso
Born with Esophageal Atresia

ten Kate, Chantal A.

ten Kate, C.A.; de Klein, A.; de Graaf, B.M.; Doukas, M.; Koivusalo, A.; Pakarinen, M.P.; van
der Helm, R.; Brands, T.; IJsselstijn, H.; van Bever, Y.; Wijnen, R.M.; Spaander, M.C.;
þÿBrosens, E. Intrinsic Cellular Susceptibility to Barrett s Esophagus in
Esophageal Atresia. Cancers 2022, 14, 513.

Article

Intrinsic Cellular Susceptibility to Barrett's Esophagus in Adults Born with Esophageal Atresia

Chantal A. ten Kate [1,2,3], Annelies de Klein [3], Bianca M. de Graaf [3], Michail Doukas [4], Antti Koivusalo [5], Mikko P. Pakarinen [5], Robert van der Helm [3], Tom Brands [3], Hanneke IJsselstijn [1], Yolande van Bever [3], René M.H. Wijnen [1], Manon C.W. Spaander [2] and Erwin Brosens [3,*]

1   Department of Pediatric Surgery and Intensive Care Children, Erasmus MC-Sophia Children's Hospital, 3000 CA Rotterdam, The Netherlands; c.tenkate@erasmusmc.nl (C.A.t.K.); h.ijsselstijn@erasmusmc.nl (H.I.); r.wijnen@erasmusmc.nl (R.M.H.W.)
2   Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, 3000 CA Rotterdam, The Netherlands; v.spaander@erasmusmc.nl
3   Department of Clinical Genetics, Erasmus MC Sophia Children's Hospital, 3000 CA Rotterdam, The Netherlands; a.deklein@erasmusmc.nl (A.d.K.); b.degraaf@erasmusmc.nl (B.M.d.G.); r.vanderhelm@erasmusmc.nl (R.v.d.H.); t.brands@erasmusmc.nl (T.B.); y.vanbever@erasmusmc.nl (Y.v.B.)
4   Department of Pathology, Erasmus MC, 3000 CA Rotterdam, The Netherlands; m.doukas@erasmusmc.nl
5   Department of Pediatric Surgery, University of Helsinki, Children's Hospital, 281, 000290 Helsinki, Finland; Antti.Koivusalo@hus.fi (A.K.); Mikko.Pakarinen@hus.fi (M.P.P.)
*   Correspondence: e.brosens@erasmusmc.nl; Tel.: +31-10-70-37643

Simple Summary: We investigated the increased prevalence of Barrett's esophagus in adults with esophageal atresia. A higher polygenic risk score and disturbances in inflammatory, stress response and oncological pathways upon acid exposure suggest a genetic susceptibility and increased induction of inflammatory processes. Although further research is required to explore this hypothesis, this could be a first-step into selecting patients that are more at risk to develop Barrett's esophagus and/or esophageal carcinoma. Currently, an endoscopic screening and surveillance program is in practice in our institution for patients born with esophageal atresia, to early detect (pre)malignant lesions. Since recurrent endoscopies can be a burden for the patient, selecting patients by for example genetic susceptibility would allow to only include those at risk in future practice.

Abstract: The prevalence of Barrett's esophagus (BE) in adults born with esophageal atresia (EA) is four times higher than in the general population and presents at a younger age (34 vs. 60 years). This is (partly) a consequence of chronic gastroesophageal reflux. Given the overlap between genes and pathways involved in foregut and BE development, we hypothesized that EA patients have an intrinsic predisposition to develop BE. Transcriptomes of Esophageal biopsies of EA patients with BE ($n = 19$, EA/BE); EA patients without BE ($n = 44$, EA-only) and BE patients without EA ($n = 10$, BE-only) were compared by RNA expression profiling. Subsequently, we simulated a reflux episode by exposing fibroblasts of 3 EA patients and 3 controls to acidic conditions. Transcriptome responses were compared to the differential expressed transcripts in the biopsies. Predisposing single nucleotide polymorphisms, associated with BE, were slightly increased in EA/BE versus BE-only patients. RNA expression profiling and pathway enrichment analysis revealed differences in retinoic acid metabolism and downstream signaling pathways and inflammatory, stress response and oncological processes. There was a similar effect on retinoic acid signaling and immune response in EA patients upon acid exposure. These results indicate that epithelial tissue homeostasis in EA patients is more prone to acidic disturbances.

Keywords: acid sensitivity; genetic predisposition; esophageal carcinoma; inflammatory response; esophagitis

1. Introduction

Esophageal atresia (EA) is a congenital foregut malformation, of which improved survival rates have resulted in a growing adult population [1]. This raises new challenges in patient care as more emphasis is placed on long-term morbidities than short-term mortality. Respiratory and gastrointestinal symptoms require long-term follow-up [2]. Many adults born with EA (EA adults) suffer from chronic gastroesophageal reflux (GER), which is often underreported by patients due to an altered perception of discomfort [3]. GER can lead to reflux esophagitis, a nonspecific inflammation of the esophagus. Furthermore, the mucosal damage resulting from GER induces the replacement of esophageal squamous epithelium by gastric columnar epithelium containing goblet cells. This precursor lesion, intestinal metaplasia (IM) also known as Barrett's esophagus (BE), can develop via dysplasia into esophageal adenocarcinoma (EAC) [4]. Basal cells at the squamous-columnar junction are the origin of the BE cell population [5]. BE tissue has crypts composed of various combinations of goblet cells, mucinous cells, endocrine cells, enterocytes and Paneth cells [6]. The prevalence of BE in EA adults is 4–5 times higher than in the general population (6.6% vs. 1.6%), and presents at a much younger median age (34 vs. 60 years) [3]. In the Erasmus MC-Sophia Children's hospital cohort, EAC has been reported in three EA patients, and—surprisingly—also esophageal squamous cell carcinoma (ESCC) is seen more frequently in patients with EA at a younger age compared with the general population [3].

Disturbances in developmental signaling pathways are often associated with metaplasia and cancer transformation. The overlap of these pathways, disease genes and risk loci for foregut morphogenesis and BE development are suggestive of a shared etiology. During embryonic development the foregut separates into the future trachea and esophagus under the influence of spatiotemporal regulated transcriptional programs. These are regulated by gradients of morphogens that lay the blueprint for their interacting cells to develop into the various esophageal cell types and structures. Six intertwined pathways are crucial in this process: TGFB-BMP, Notch, FGF, WNT, Hedgehog and retinoic acid (RA) signaling [7]. TGFB-BMP signaling [8], SHH signaling [9] as well as RA signaling [10] are dysregulated in BE. Additionally, genome-wide association studies (GWAS) describe risk loci for the development of BE, EAC and ESCC near genes involved in these foregut developmental genes and pathways. These include *TBX5*, *GDF7*, *CRTC1*, *BARX1*, *FOXP1* and *FOXF1* [11].

Given the increased incidence of BE in EA adults, endoscopic surveillance is recommended [12]. Surveillance leads to early detection of BE or esophageal carcinoma, but could also create an unnecessary burden of repeated endoscopies for those not at risk as well as substantial added health care costs. Identifying patients at risk for developing BE could be a first step towards a tailor-made surveillance strategy. In this study, we hypothesize that patients born with EA have an increased (genetic) susceptibility for BE development. We aim to identify this predisposition by comparing risk loci burden and transcriptomes of patients with EA who have developed BE with EA patients without BE, and patients with BE without an EA history. We show that in both groups BE is histopathologically similar. However, the effect of acid reflux seems different with intrinsic cellular differences in inflammatory and stress response pathways, RA metabolism and signaling.

2. Materials and Methods

2.1. Study Population

Our institutional review board approved this case-control study (MEC-2018-1500). In our surveillance program, patients undergo upper endoscopies with histologic evaluation of biopsies taken according to a standardized protocol [3]. Biopsies and blood used in this study were retrieved from the Biobank Esophageal Atresia (MEC-2015-645) and the Biobank Barrett (MEC-2010-094). Mucosal esophageal biopsies were taken from two sites: (1) unaffected esophageal squamous cell epithelium (SQ), in EA patients taken above the original anastomosis; and (2) the GEJ or—if present—from Barrett's mucosa. Sample extraction protocol and storage are described in Supplementary Methods SM1. Additionally, we genotyped six EA/BE patients from a Finnish cohort study (447/E7/2005) [13], as well as

730 ancestry matched (broadly European) unaffected controls. For the in vitro experiments we used human fibroblasts from EA patients and healthy controls. EA fibroblast lines were taken during routine diagnostic procedures. Control fibroblast lines are anonymized lines that taken previously during unrelated routine diagnostic procedures and stored for research purposes. We compared three groups of patients: patients with EA who have developed BE (EA/BE), patients with EA without BE (EA-only), and patients with BE without EA in history (BE-only) BE-only patients were matched for age and gender with EA/BE patients. See Figure 1 for study set-up.
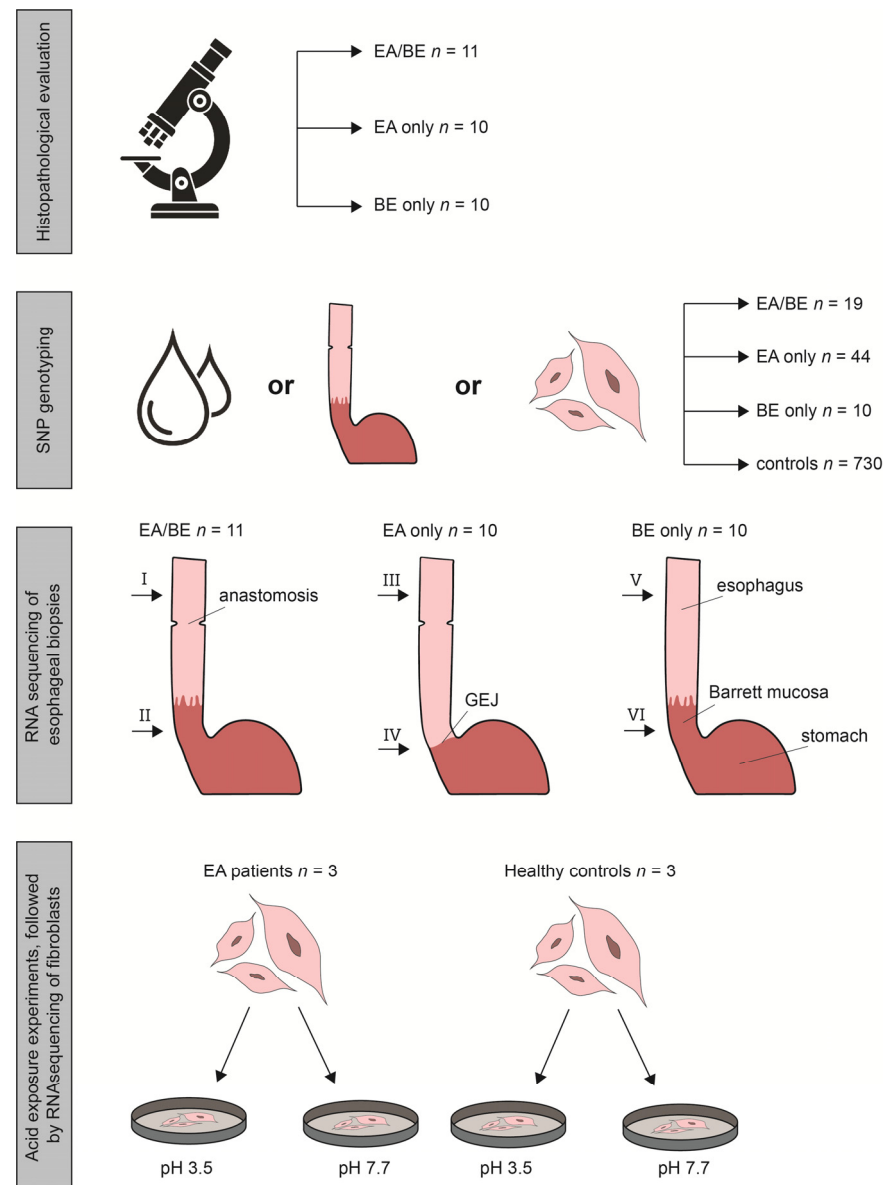
Figure 1. Schematic overview of the study set-up and number of patients included in each part. We compared three groups of patients: patients with esophageal atresia (EA) who have developed Barrett's esophagus (BE, EA/BE), patients with EA without BE (EA-only), and patients with BE without EA in history (BE-only). BE-only patients were matched for age and gender with EA/BE patients. Roman numerals I to VI indicate the subgroups, based on the location of the biopsies. GEJ = gastroesophageal junction.

2.2. Histopathological Evaluation

Hematoxylin and eosin-stained histological slides were retrieved from the archives of all patients of whom biopsies had been collected for RNA sequencing. All slides were blinded reassessed by a BE expert pathologist, according to a review-based checklist [6]. Potential differences were scored between the three groups.

2.3. SNP Genotyping and Calculation of Predisposing SNPs, Associated with BE

DNA extraction and quantification was done according standard procedures (see Supplementary Methods SM2). Processing of the SNP array genotyping chips (Infinium Global Screening Array v1.0 or v3.0 Illumina, Inc., San Diego, CA, USA) was done according to the manufacturer's standard protocol (SM3). Output was generated using Illumina Genome studio v2.0 (Illumina, San Diego, CA, USA). Predisposition loci (and corresponding lead or proxy SNPs) associated with BE, EAC and/or ESCC were derived from literature. We used genotype data from EA/BE patients ($n = 19$), EA-only patients ($n = 44$), BE-only patients ($n = 10$) and controls ($n = 730$) to see if previously BE associated SNPs were more prevalent in EA/BE patients (see Supplementary Methods SM3). We used the allele counts and published ORs of the associated SNPs to calculate a polygenic risk score (PGRS) using an additive model: PGRS = $\sum$ Ln (OR risk allele) $\times$ allele count (see Supplementary Tables S1–S5) Since we do not know if these ORs are precise enough to calculate the risk for the combination of EA and BE, we used the ORs of the associated SNPs calculated from our study population in a second calculation (see Supplementary Table S6). Using a Kruskal–Wallis test and Mann–Whitney tests, we compared the PGRS between the different groups. All statistical analyses were performed in SPSS V.25.0 (IBM, Chicago, IL, USA), with a significance level of $p < 0.05$.

2.4. RNA Sequencing, Differential Gene Expression and Pathway Enrichment Analysis

RNA extraction and quantification was done according standard procedures (see Supplementary Methods SM2). Genome-wide individual gene expression raw counts are available in Supplementary Datafile S1. Differential expression was calculated between (sub)groups (see Supplementary Methods SM4). Genes with a maximum group mean > 2, a fold change $\geq$ 1.5 and a false discovery rate (FDR) *p*-value < 0.05 were considered significantly differentially expressed. All differentially expressed genes per subgroup analysis were uploaded into the Ingenuity Pathway Analysis (IPA) software (Qiagen, Venlo, The Netherlands). Core analysis was performed for each (sub)group. A *p*-value of <0.05 and a Z-score of $\geq$2 were considered significant. Our ethics committee does not allow sharing of individual patient or control genotype information in the public domain, including sequencing reads.

2.5. Acid Exposure Experiments

In absence of available epithelial cells for in-vitro studies we used fibroblast. Activated fibroblasts generate extracellular matrix components and regulate inflammation [14]. There are several lines of evidence supporting a role for fibroblasts in BE proliferation and cancer [15,16]. To simulate a one-time acid reflux episode on RNA level, human fibroblasts from EA patients ($n = 3$) and healthy controls ($n = 3$) were exposed to pH adjusted cell culture medium conditions (see Supplementary Methods SM5). Hydrochloric acid was added to culture medium until the desired pH level was reached. Subsequently, cells were washed with phosphate buffered saline (PBS) and given standard medium. After 24 h, survival was measured (see Supplementary Table S7) with the TC20™ Automated Cell Counter (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Cell morphology was evaluated (see Supplementary Figure S1) with the Olympus IX70-S8F Inverted Fluorescence Microscope (Olympus Corporation, Tokyo, Japan). RNA was isolated and sequenced as described in Supplementary Methods SM2 and SM4. Expression levels were compared with the RNA sequencing results of the esophageal biopsies.

2.6. Study Approval

The Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved this study (MEC-2015-645, MEC-2010-094, MEC-2012-387). All authors had access to the study data and reviewed and approved the final manuscript.

3. Results

3.1. Study Population

Patient characteristics are depicted in Supplementary Tables S8 and S9. Histopathological assessment (see Supplementary Figure S2) of the biopsies is summarized in Supplementary Table S10. Columnar epithelium was present in all groups, except for two EA-only patients (see Supplementary Table S11). Since EA-only patients were selected as not having metaplasia in the distal esophagus at endoscopy, this means that most biopsies could contain part of the cardia as well. Neutrophil granulocytes were absent in the majority of EA-only patients, while a varying degree of nonspecific inflammatory cell infiltrate was present in most of them. Focusing on the characteristics of BE, IM with the presence of goblet cells was similarly present in EA/BE patients and BE-only patients. The amount of IM was larger in BE-only patients. No dysplasia was found in any of the samples.

3.2. SNP (Single Nucleotide Polymorphism) Genotyping

Given the limited sample size of our study population, we used ORs selected from literature to calculate the contribution of predisposing associated SNPs (polygenetic risk score, PGRS). Supplementary Table S1 depicts an overview of the included SNPs and ORs. Using these ORs, we found a median PGRS of 3.24 (range 1.39–4.68) for EA/BE patients, of 2.98 (1.19–4.74) for EA-only patients and of 2.63 (1.85–3.53) for BE-only patients. There were no statistical significant differences between these groups (Figure 2A, panel a, all $p > 0.05$). When using our own data, we did find significant differences in PGRS between these groups (Figure 2A, panel b). A higher risk allele frequency was found for EA/BE patients versus BE-only patients for rs3784262 near ALDH1A2 ($p = 0.017$), and a lower risk allele frequency of rs3072 near GDF7 ($p = 0.009$) (Figure 2B and Supplementary Table S3).

3.3. RNA Sequencing of Esophageal Biopsy Specimens

An average of 88,378,214 reads per sample were generated (62,471,354–165,874,334). Of these reads, 98% (94.9–98.4) aligned to the human reference genome. A total of 9752 transcripts had a mean expression of ≥ 2 RPKM and were considered expressed. See Supplementary Tables S12–S14 for the quality reports. PCA of the gene expression data confirmed clustering of the samples into the three groups (see Supplementary Figures S3 and S4). PCA and quality control procedures included the exclusion of two outliers (BBE-017 and BBE-079).

3.4. Differential Expression and Pathway Enrichment Analysis of Esophageal Biopsy Specimens

Seven known BE disease genes [11] were differentially expressed between EA-only patients and EA/BE or BE-only patients (Figure 3 and Supplementary Table S15). Enriched pathways between EA/BE patients and BE-only patients were involved in RA signaling, stress response and inflammatory pathways, and oncological processes (see Figure 4 and Supplementary Table S16).
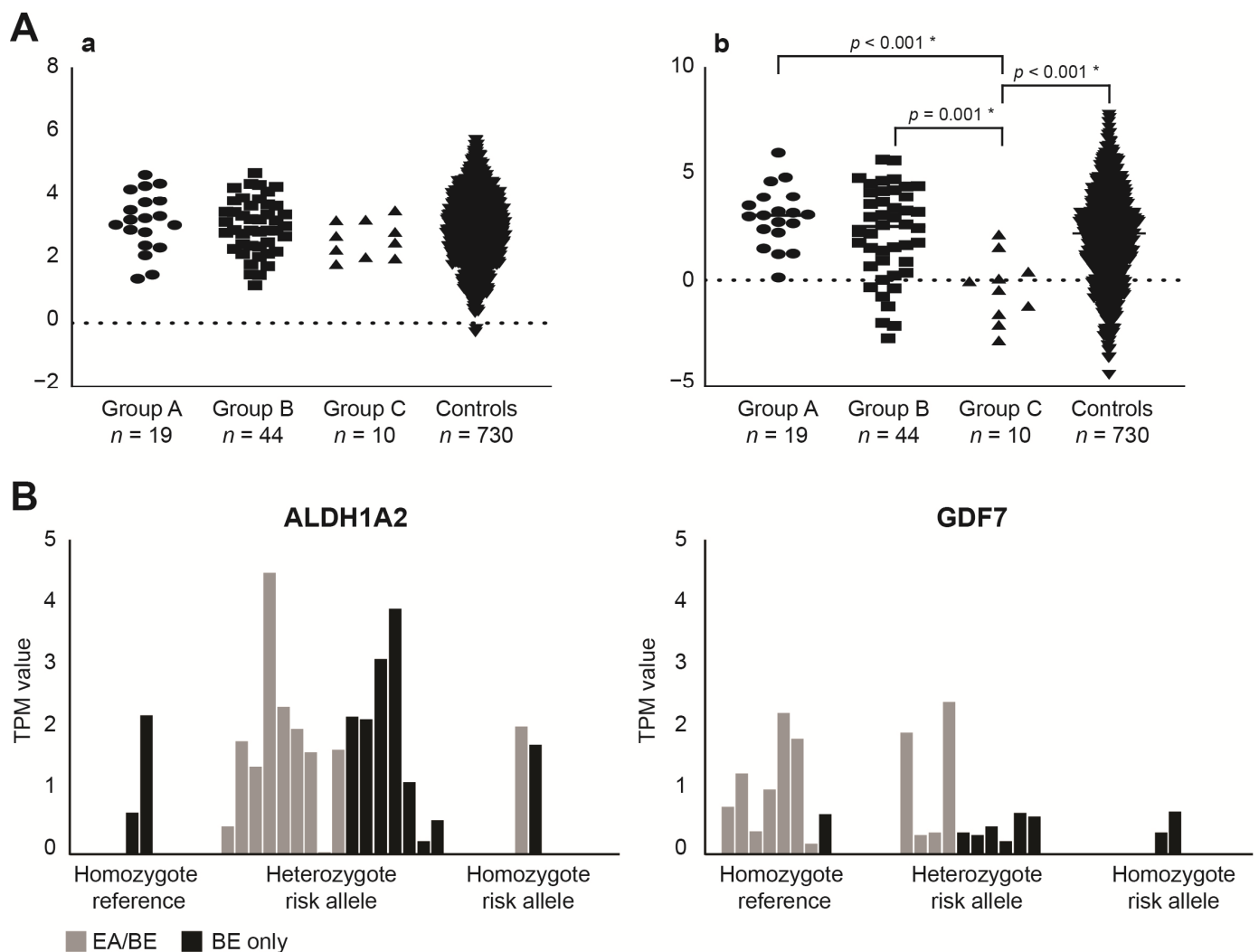
Figure 2. (**A**) Polygenic risk scores (PGRS) per patient. Group A = patients with esophageal atresia (EA) and Barrett's esophagus (BE), group B = patients with EA without BE, group C = patients with BE without EA in history. Panel a (left) are PGRS based on odds ratios (ORs) selected from the literature. No statistical significant differences between the groups were observed. Panel b (right): PGRS based on ORs calculated from our study population. We found a median PGRS of 3.05 (range 0.14–6.04) for EA/BE patients, of 2.52 (−2.73–5.72) for EA-only patients and of −0.24 (−2.83–2.15) for BE-only patients. A Kruskal–Wallis test revealed a significant difference in PGRS based on ORs calculated from our study population between the four groups ($p = 0.001$). T-statistics indicated a difference between BE-only patients versus EA/BE patients ($p < 0.001$), EA-only patients ($p = 0.001$) and controls ($p < 0.001$). Asterisk (*) indicates significance $p < 0.05$. (**B**) Gene expression levels for ALDH1A2 and GDF7 per patient, sorted based on the genotype of the patients. A higher risk allele frequency was found for EA/BE patients versus BE-only patients for rs3784262 near ALDH1A2 ($p = 0.017$) and a putative protective allele for rs3072 near GDF7 ($p = 0.009$). Looking at gene expression levels, GDF7 has slightly elevated TPM values for patients homozygote for the reference allele. No significant differences could be detected for these two associated SNPs. TPM = transcripts per million, EA = esophageal atresia, BE = Barrett's esophagus. Complete results can be found in Supplementary Tables S3–S6.
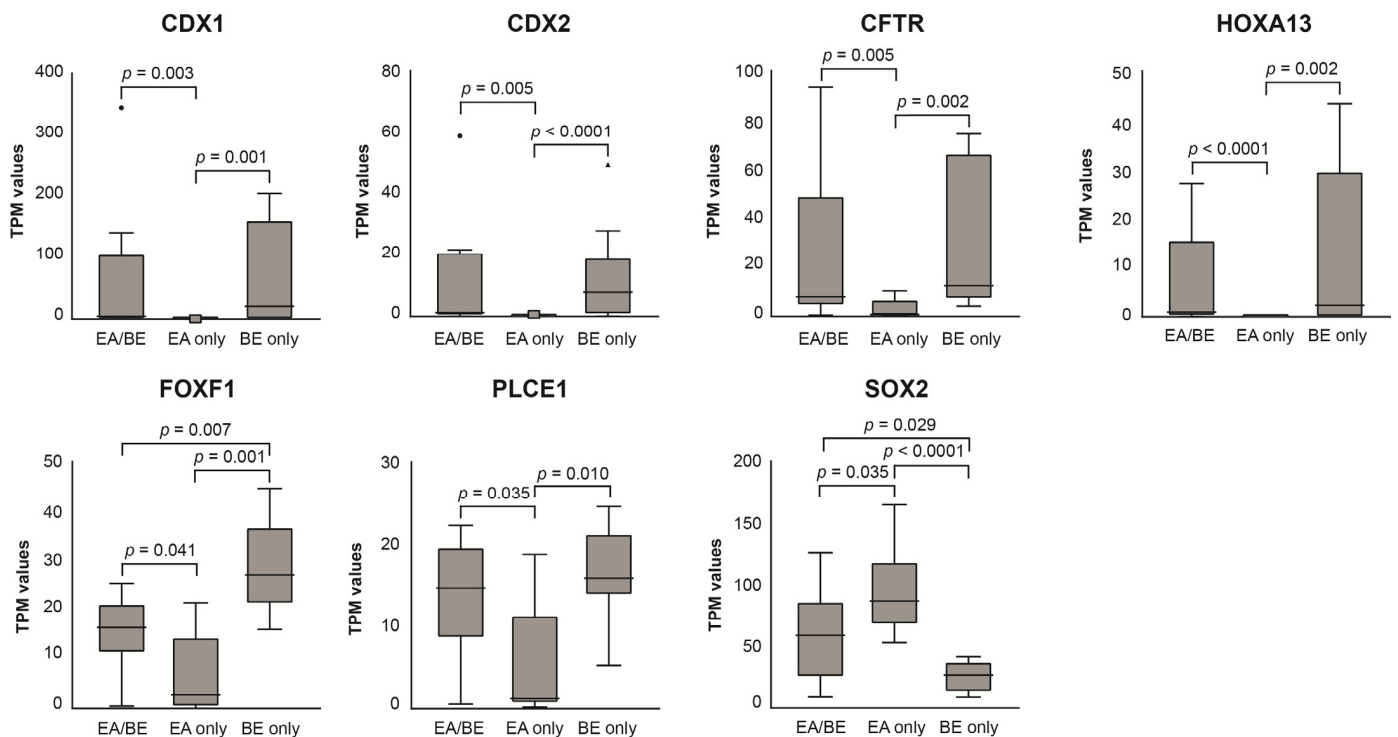
Figure 3. Gene expression levels per group for selected disease genes, involved in foregut morphogenesis and/or associated with Barrett's esophagus in literature, presented as median (interquartile range) with minimum and maximum values. We compared biopsies of the gastroesophageal junction between three groups of patients: patients with esophageal atresia (EA) who have developed Barrett's esophagus (BE) (EA/BE, *n* = 11), patients with EA without BE (EA-only, *n* = 10), and patients with BE without EA in history (BE-only, *n* = 10). TPM = transcripts per million, EA = esophageal atresia, BE = Barrett's esophagus.

3.5. Acid Exposure Experiments

To study the effect of GER on RNA level, we simulated a reflux episode in in vitro experiments (see Figure 1). First, we optimized the acid exposure experiment (see Supplementary Methods SM5). Next, we exposed fibroblasts from three EA patients and three healthy controls for 30 min to medium with pH 3.5 or to normal medium (control). Cells exposed to pH 3.5 showed cell rounding and irregular cell membranes (see Supplementary Figure S5). After acid exposure, there was a clear difference between upregulated and down-regulated genes, both in patients and controls (see Supplementary Figure S6). Ten pathways were enriched with differentially expressed genes between patients and controls (see Supplementary Table S17), that contained 244 differentially expressed genes. Subtracting the genes that were also differentially expressed without acid exposure, 81 genes of interest remained (see Supplementary Figure S7). Pathway analysis of these 81 genes confirmed enrichment of pathways mostly involved in inflammatory processes (see Supplementary Table S18). Finally, we compared the results of the pathway analysis of the biopsies with those of the fibroblasts after acid exposure. Of the enriched pathways between GEJ samples of EA/BE patients and BE-only patients, 20 pathways were also enriched between fibroblasts of EA patients and controls after acid exposure (Table 1. In total, seven genes within these pathways were differentially expressed in both the GEJ samples and the acid-exposed fibroblasts (see Supplementary Figure S8).
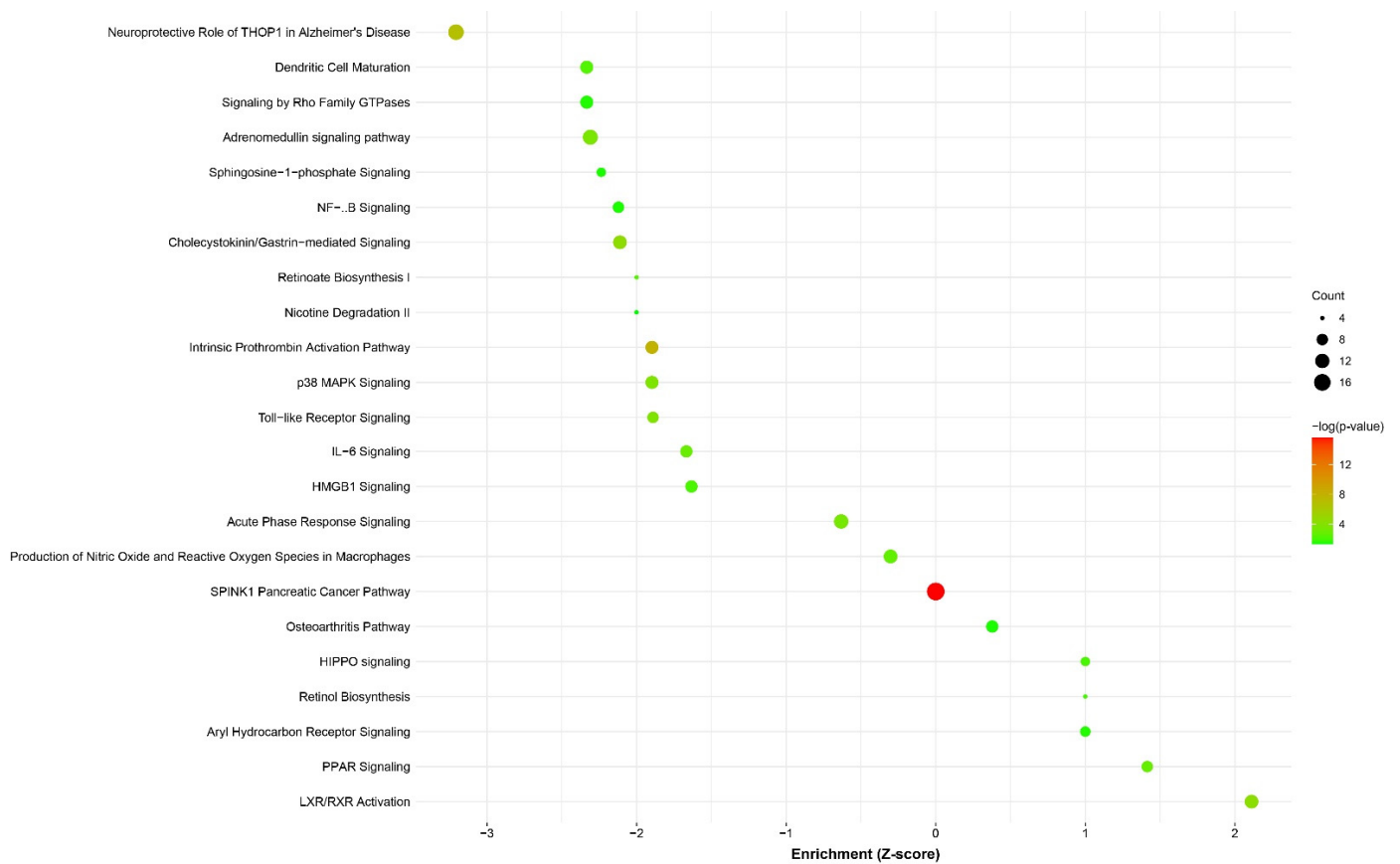
Figure 4. Bubble plot of canonical pathways, significantly enriched by differentially expressed genes, between gastroesophageal junction (GEJ) samples of group A (esophageal atresia (EA) with Barrett's esophagus (BE)) and GEJ samples of group C (BE-only). The color and size of the dots represent the range of the *p*-value and the number of molecultes mapped to the indicated pathways. Settings: *p*-value < 0.05 (=−log(*p*-value) > 1.3), z-score < −2 or >2. SPINK1 Pancreatic Cancer Pathway is also the only significantly upregulated pathway, when comparing group A (EA/BE) with group C (BE-only). Plotted by http://www.bioinformatics.com.cn (accessed on 24 November 2021), a free online platform for data analysis and visualization.

Table 1. Overlap between canonical pathways, significantly enriched by differentially expressed genes, in the esophageal biopsy specimens versus the acid-exposed and non-exposed fibroblasts. II = gastroesophageal junction (GEJ) samples from group A (esophageal atresia (EA) and Barrett's esophagus (BE)), VI = GEJ samples from group C (BE-only). *n* = total number of canonical pathways significantly enriched by differentially expressed genes. N/A = not applicable, z-score could not be calculated. Grey box indicates that a pathway was not present in the results of that pathway analysis.

	Esophageal Biopsy Specimens		Fibroblasts from Acid Exposure experiment					
	II vs. VI (*n* = 353)		EA Patients vs. Controls (Acid-Exposed) (*n* = 258)		EA Patients vs. Controls (Non-Exposed) (*n* = 314)		Acid-Exposed vs. Non-Exposed (All Samples) (*n* = 578)	
Canonical Pathways	$-log(p\text{-}Value)$	Z-Score	$-log(p\text{-}Value)$	Z-Score	$-log(p\text{-}Value)$	Z-Score	$-log(p\text{-}Value)$	Z-Score
Agranulocyte Adhesion and Diapedesis	1.69	N/A	-	-	1.52	N/A	-	-
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	3.22	N/A	2.57	N/A	2.05	N/A	-	-
Atherosclerosis Signaling	4.93	N/A	2.04	N/A	2.23	N/A	-	-
Cholecystokinin/Gastrin-mediated Signaling	4.38	2.111	2.35	0	1.39	N/A	-	-
Communication between Innate and Adaptive Immune Cells	2.39	N/A	2.47	N/A	-	-	-	-
Dendritic Cell Maturation	2.27	2.333	4.600	−0.707	2.19	−1.633	-	-
Extrinsic Prothrombin Activation Pathway	1.36	N/A	2.34	N/A	-	-	-	-
Glucocorticoid Receptor Signaling	4.51	N/A	1.53	N/A	2.03	N/A	-	-
Graft-versus-Host Disease Signaling	4.23	N/A	3.600	N/A	-	-	-	-
HMGB1 Signaling	2.09	1.633	2.37	N/A	-	-	-	-
IL-6 Signaling	2.89	1.667	1.33	N/A	-	-	5.02	2.117
Intrinsic Prothrombin Activation Pathway	7.92	1.897	2.61	N/A	-	-	-	-
LXR/RXR Activation	4.31	−2.111	2.12	−1	-	-	-	-
MSP-RON Signaling Pathway	4.58	N/A	1.44	N/A	1.81	N/A	-	-
Osteoarthritis Pathway	1.44	−0.378	-	-	6.98	−1.265	-	-
PPAR Signaling	2.81	−1.414	2.33	0	-	-	2.57	−2.524
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	2.68	0.302	1.48	N/A	-	-	-	-
Retinol Biosynthesis	1.95	−1	1.49	N/A	-	-	-	-
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.59	N/A	3.710	N/A	3.87	N/A	-	-
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	3.10	N/A	3.280	N/A	4.53	N/A	-	-
Sphingosine-1-phosphate Signaling	1.44	−2.236	1.40	N/A	2.38	−1	-	-

4. Discussion

In this first translational case-control study in adults born with esophageal atresia (EA), we compared EA patients who developed Barrett's esophagus (BE, EA/BE) to EA patients who did not develop BE (EA-only) and BE patients without a history of EA (BE-only). Previous studies described an increased prevalence of BE in EA adults—and at a much younger age—compared with the general population [3]. Over the years, several risk loci associated with BE and/or esophageal carcinoma have been published, of which many near genes involved in foregut development [11] (S2). This overlap made us hypothesize that EA patients have an increased (genetic) susceptibility to develop BE.

4.1. BE Characteristics of EA/BE Patients and BE-Only Patients

There is a twenty-year difference in the age at which biopsies were taken between EA/BE patients and BE-only patients. We confirmed the lack of morphological differences between these two groups. Although endoscopic esophagitis was absent in the majority of the BE-only patients, neutrophil granulocytes were present in these patients. The typical characteristics of BE (columnar metaplasia with presence of goblet cells) were equally present, although the larger amount of IM in BE-only patients is indicative of a more advanced stage. Paneth cells were present in some patients of both groups, a variety more often reported in BE [6].

4.2. The Contribution of BE Associated SNPs in EA/BE Patients

The overlap of genes involved in foregut development and risk loci for BE insinuates a genetic predisposition for EA patients to develop BE. For example, *FOXF1*, which is expressed in the developing foregut [17], *BARX1*, which is expressed at the tracheoesophageal separation site and inhibits Wnt signaling [18], and *FOXP1*, which regulates esophageal muscle development [19], have all been associated with BE in previous GWAS studies [11]. *FOXP1* has also been implicated as a tumor suppressor gene in several tissues including the gastrointestinal tract [20]. The ORs of these risk loci were often small and the GWAS studies included large sets of BE patients in order to detect these predispositions.

Regardless, there seems to be an elevated risk for EA patients. EA/BE patients have a higher median PGRS compared with BE-only patients (3.24 vs. 2.63, $p = 0.069$), which was confirmed and reached significance when using ORs calculated from our study population ($p < 0.001$, see Figure 2A and Supplementary Tables S4 and S6). Despite the small cohorts, the higher PGRS in EA/BE patients is suggestive for an increased predisposition, and a possible contribution for the earlier age of onset of BE in these patients. Such a relationship (higher PGRS and earlier disease onset) has been demonstrated previously in patients with atrial fibrillation [21]. However, differences in PGRS are not likely to be sufficient on their own to exclude EA patients from (pre)malignant screening protocols. Ideally, a screening algorithm would contain multiple risk factors of which the PGRS could be one. Further research would be required to confirm the impact of risk loci for BE and their potential benefit in surveillance strategies for EA patients.

Two predisposing associated SNPs proved enriched when comparing EA/BE patients with BE-only patients: rs3784262 near *ALDH1A2* (OR 3.94, $p = 0.017$) and rs3072 near *GDF7* (OR 0.22, $p = 0.009$). *ALDH1A2* (also known as *RALDH2*) is an enzyme that catalyzes the transformation of retinaldehyde into RA, a key morphogen in foregut development [22]. Lack of RA signaling results in increased TGFB-BMP signaling and hampers lung bud induction [23]. In contrast, BE is characterized by a higher expression of this enzyme, resulting in higher levels of RA [24]. *GDF7* is also a component of the TGFB-BMP signaling pathway. TGFB-BMP signaling is essential in esophageal formation by inhibiting *SOX2* in the ventral foregut [25] but also contributes to the differentiation of columnar epithelium and BE development by interacting with *CDX1* and *CDX2* [26]. Interestingly, the associated SNP *GDF7* seems a protective locus in EA/BE patients (OR 0.22, $p = 0.009$). The trends shown by these results are illustrative but more research is needed. Though EA/BE

patients could have an increased genetic risk, the current sample sizes do not allow to draw firm conclusions.

4.3. EA/BE Patients Have Comparable Gene Expression of BE Disease Genes as BE-Only Patients

An earlier age of BE onset in EA patients could mean that epithelial homeostasis in these patients is more prone to disturbances. To investigate this, we sequenced RNA extracted from esophageal biopsies of three groups (EA/BE, EA-only and BE-only). We evaluated the expression of BE disease genes but found no difference in expression between EA/BE patients and BE-only patients. In both groups, these genes were upregulated compared to EA-only patients, indicating that the BE found in EA/BE patients is similar to the BE in BE-only patients.

4.4. EA/BE Patients Have an Increased Inflammatory Response

Since the expression of disease genes could not explain the earlier age of onset, we explored the complete transcriptome and corresponding differentially expressed genes and pathways. Many of the enriched pathways in EA/BE patients compared with BE-only patients, hinted at upregulated inflammatory (e.g., IL-6 signaling) and stress response pathways, downregulated oncological processes and dysregulated RA signaling (see Supplementary Table S16). Inflammatory cells produce carcinogenic compounds that can initiate DNA damage. The secretion of growth factors and cytokines increase proliferation and transition to tumor cells [27]. *SPINK1* expression itself has the potential to be a BE biomarker as it lacks expression in unaffected esophageal tissue (see Supplementary Figure S9).

Human studies and in vitro experiments have shown that exposure of esophageal tissue to low pH and/or bile acids may induce cell proliferation and reduce cell apoptosis through an increased expression of cyclo-oxygenase-2 (COX-2), prostaglandin E_2 (PGE2), mitogen-activated protein kinase (MAPK) and NF-κB pathways [28–31]. In our data, p38 MAPK Signaling and NF-κB Signaling are upregulated in EA/BE patients compared with BE-only patients. Given their proliferative and anti-apoptotic role, these pathways could be valuable for BE staging. Quante and coworkers showed that transgenic mice, overexpressing human *IL-1β*, presented with chronic inflammation, BE and esophageal dysplasia. Oral exposure to bile acids led to elevated IL-6 levels, accelerating BE development and progression into EAC, and implicating an IL-1β-IL-6 signalling cascade [32]. Clinical management of BE is focused around chemical inhibition of acid exposure and decrease of inflammation. Inhibition of gastric acid secretion with proton pump inhibitors (PPIs) reduces the transition to dysplasia in BE patients [33] and a combination of non-steroidal anti-inflammatory drugs (NSAIDs) and statins may reduce neoplastic progression [34]. Recently, it has been shown that the combination of high-doses esomeprazole and aspirin reduces high-grade dysplasia and EAC in BE patients [35]. Given the potentially altered response to acid in EA patients, the effectiveness of PPIs and NSAIDs in this population warrant further investigation.

Furthermore, stress response pathways are upregulated. Cholecystokinin/Gastrin-mediated Signaling is an activator of actin stress fiber formation and intertwined with stress response pathways as p38 MAPK Signaling, Sphingosine-1-phosphate Signaling and Signaling by Rho Family GTPases. These processes may lead to the conversion of squamous epithelium to columnar metaplasia. Another study showed that low pH and/or bile acids can induce oxidative stress, which causes DNA damage [36]. In combination with reduced apoptosis this can lead to dysplasia. When this is followed by neoplastic progression BE can develop into EAC.

4.5. Dysregulation of RA Metabolism and Signaling

RA is increased in BE and works—like bile acids—through the RXR receptors to transform squamous epithelium to columnar epithelium [10]. LXR/RXR activation, involved in RA mediated gene activation, is downregulated in EA/BE patients compared

with BE-only patients. Retinol biosynthesis is also downregulated, whilst its downstream processes in all trans RA synthesis (Retinoate Biosynthesis I) are upregulated. Peroxisome proliferator-activated receptors (PPARs) are transcription factors activated by RA, generally upregulated in BE [37], but downregulated in EA/BE patients. Like discussed above, the downregulation of these pathways could indicate that BE-only patients are at a more advanced stage than EA/BE patients. Given the clinical differences (age and length of BE) between these patients, this does make sense.

4.6. Downregulation of the Hippo/YAP Pathway

Downregulation of oncological pathways in EA/BE patients could be indicative of either a decreased progression rate to dysplasia or a less advanced state of progression compared with BE-only patients. The Hippo/YAP pathway is important in cell proliferation, survival, and differentiation. Yes-association protein (YAP) expression is associated with dysplasia and adenocarcinoma [38]. Hippo signaling is involved in cell contact inhibition [39] as is Aryl Hydrocarbon Receptor Signaling [40]. Hippo activation (and YAP inactivation) is necessary for programmed cell death after detachment from the extracellular matrix [41]. Therefore, downregulation of this pathway could (in theory) decrease anoikis and increase the risk of tumor cell metastasis.

4.7. EA Patients Seem to Be More Sensitive to Acid Reflux Exposure

EA patients are earlier in life and more frequently exposed to GER. Chronic GER could be a consequence of the surgical repair: the lower esophageal sphincter is often retracted above the diaphragm, resulting in the loss of the natural reflux barrier function of the GEJ [42]. Other factors contributing to GER are impaired motility, delayed bolus clearance and delayed gastric emptying [43]. There seems to be a direct relationship of these symptoms with EA, as Adriamycin induced EA rats have impaired esophageal relaxation and a decreased number of ganglia and nerve fibers in the esophageal myenteric plexus [44]. The prevalence of mucosal damage is related to the level of pH exposure and to the composition of the acid reflux [45]. Animal studies have shown that acid fluids can activate pepsin, which inflicts injury and leads to mucosal damage [46].

We speculated that GER could result in an upregulation of inflammatory pathways. Additionally, EA patients could have a predisposition that makes them more sensitive to acid reflux than the general population. To explore these hypotheses, we performed in vitro experiments to simulate a one-time reflux episode in fibroblasts of EA patients and healthy controls. The enriched pathways of the GEJ biopsies of EA/BE patients showed an overlap with the enriched pathways of the fibroblasts of EA patients after acid exposure— but not with those of healthy controls. These overlapping pathways were again mostly involved inflammatory or oncological processes. For example, LXR/RXR Activation, PPAR Signaling and Retinol Biosynthesis were also enriched in fibroblasts of EA patients after acid exposure, hinting at intrinsic disturbances of RA signaling in EA patients under the influence of GER.

We do not know of the three patients used in the in vitro experiment will develop BE in time as the fibroblasts are derived of patients currently aged 29, 30 and 39 years old. It is, however, interesting that we could detect a similar predisposition in just 3 EA patients, and as a general response (in fibroblasts) to acid.

4.8. Strengths and Limitations

The main strength of this study is the broad investigative approach by combining histology, genotype, transcriptome and in vitro results. Some limitations should be addressed. First, due to the relative low incidence of EA and corresponding small sample sizes, we mostly observed trends and more EA/BE patients are needed to draw more robust conclusions. At this point, the difference in gene expression between EA/BE patients and EA-only patients is negligible. This could be due to the fact that most biopsies could contain part of the cardia. However, the power would increase substantially if we would know

which EA patients have not developed BE throughout their life, as the current EA-only population is a mixture of patients who have not yet and will never develop BE. Second, EA is a heterogeneous disease. Our study population included both patients with isolated EA and patients with syndromes or multiple anomalies. This phenotypic heterogeneity might also be the results of a genetic heterogeneity. Thirdly, BE can present as a heterogeneous metaplastic mosaic, consisting of multiple individual crypts that arose from independent clones [47], which have distinct ploidies, copy number variations (CNV) and point mutations [48]. Heterogeneity in these crypts pose a risk of sampling error. Even within long segment BE, IM can be focally distributed [49]. Recent progress in genetic analysis of BE stem cells and EAC indicates that there are patient-specific driver genes affected in both the precursor lesion [50] and subsequent cancer of the esophagus [51]. Perhaps the heterogeneous background of de novo mutations [52] and de novo CNVs [53] in EA contributes to this patient-centred susceptibility. This could have created larger variances in gene expression per evaluated group. Subsequent experiments using single-cell sequencing of definite IM could reveal differences between patients that cannot be detected in whole biopsy specimens. Lastly, morphological differences were absent. However, segment length differences could be related to a difference in disease stage [54] and impact gene networks are prone to disturbances.

5. Conclusions

Altered regulation of p38 MAPK, NF-κB and RA signaling could have implications for (or be related to) the dysplastic progression. If Hippo/YAP signaling remains downregulated upon progression to cancer, the metastasis risk could be higher in EA patients due to reduced anoikis. An increased PGRS and upregulation of inflammatory pathways hint at a multifactorial contribution underlying the earlier age of onset of BE in EA patients. We did not evaluate mechanical factors such as loss of the natural reflux barrier due to the surgical repair and clinical factors such as impaired esophageal motility. These factors increase the level of acid exposure and likely add to the effect of risk loci and primed inflammatory pathways.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers14030513/s1, Table S1: Genes and polymorphisms associated with Barrett's esophagus (BE), with selected proxy SNPs used for SNP array genotyping. Table S2: Genes and polymorphisms associated with Barrett's esophagus (BE), esophageal adenocarcinoma (EAC) or esophageal squamous cell carcinoma (ESCC). Table S3: Odds ratios calculated from the single nucleotide polymorphism (SNP) genotyping data. Table S4: Overview of polygenic risk scores (PGRS) for all groups. Table S5: Overview of the selected odds ratios (OR) used for the polygenic risk score. Table S6: Comparison of all groups separately for the polygenic risk score (PGRS). Table S7: Overview of survival rates of fibroblast cells after exposure to pH adjusted medium. Table S8: Basic characteristics of selected patients and controls for RNA sequencing of the esophageal biopsy specimen and for the SNP array genotyping. Table S9: Phenotype description. Table S10: Summarized results of reassessments of pathology slides of esophageal biopsy specimens. Table S11: Complete results of reassessments of pathology slides of esophageal biopsy specimens. Table S12: Results of RNA and DNA isolation. Table S13: Quality report of RNA sequencing data from esophageal biopsy specimens. Table S14: Results of RNA isolation from fibroblast of the acid exposure experiments. Table S15: Number of significantly differently expressed genes when comparing the different subgroups. Table S16: Canonical pathways, significantly enriched by differentially expressed genes, and corresponding diseases and bio functions. Table S17: Canonical pathways, enriched by differentially expressed genes. Table S18: Canonical pathways, significantly enriched by differentially expressed genes. Figure S1. Overview of survival rates of fibroblast cells after exposure to pH adjusted medium. Figure S2: Examples of the review-based checklist used for the histopathological assessments. Figure S3: Two-dimensional scatter plot of principal component analysis (PCA). Figure S4: Tree-dimensional scatter plot of principal component analysis (PCA). Figure S5: Morphology (20× magnification) of fibroblast cells. Figure S6: Heat map of mean transcript per million (TPM) for fibroblast cells after the in vitro experiment. Figure S7: Number of differentially expressed genes between patients with EA

and healthy controls. Figure S8: Violin plots of gastroesophageal (GEJ) samples and box plots of acid-exposed fibroblasts. Figure S9. SPINK1 is a potential BE biomarker. Datafile S1: Individual gene counts (Excel sheet). SM1: Sample extraction protocol and storage. SM2: RNA and DNA isolation. SM3: SNP genotyping. SM4: RNA sequencing. SM5: Acid exposure experiments. References [55–78] are cited in the supplementary materials.

References

1. Wang, B.; Tashiro, J.; Allan, B.J.; Sola, J.E.; Parikh, P.P.; Hogan, A.R.; Neville, H.L.; Perez, E.A. A nationwide analysis of clinical outcomes among newborns with esophageal atresia and tracheoesophageal fistulas in the United States. *J. Surg. Res.* **2014**, *190*, 604–612. [CrossRef] [PubMed]
2. Van Lennep, M.; Singendonk, M.M.J.; Dall'Oglio, L.; Gottrand, F.; Krishnan, U.; Terheggen-Lagro, S.W.J.; Omari, T.I.; Benninga, M.A.; van Wijk, M.P. Oesophageal atresia. *Nat. Rev. Dis. Primers* **2019**, *5*, 26. [CrossRef]
3. Vergouwe, F.W.T.; IJsselstijn, H.; Biermann, K.; Erler, N.S.; Wijnen, R.M.H.; Bruno, M.J.; Spaander, M.C.W. High Prevalence of Barrett's Esophagus and Esophageal Squamous Cell Carcinoma After Repair of Esophageal Atresia. *Clin. Gastroenterol. Hepatol.* **2018**, *16*, 513–521.e6. [CrossRef]
4. Solaymani-Dodaran, M.; Logan, R.F.; West, J.; Card, T.; Coupland, C. Risk of oesophageal cancer in Barrett's oesophagus and gastro-oesophageal reflux. *Gut* **2004**, *53*, 1070–1074. [CrossRef]
5. Jiang, M.; Li, H.; Zhang, Y.; Yang, Y.; Lu, R.; Liu, K.; Lin, S.; Lan, X.; Wang, H.; Wu, H.; et al. Transitional basal cells at the squamous-columnar junction generate Barrett's oesophagus. *Nature* **2017**, *550*, 529–533. [CrossRef] [PubMed]
6. Naini, B.V.; Souza, R.F.; Odze, R.D. Barrett's Esophagus: A Comprehensive and Contemporary Review for Pathologists. *Am. J. Surg. Pathol.* **2016**, *40*, e45–e66. [CrossRef]
7. Han, L.; Chaturvedi, P.; Kishimoto, K.; Koike, H.; Nasr, T.; Iwasawa, K.; Giesbrecht, K.; Witcher, P.C.; Eicher, A.; Haines, L.; et al. Single cell transcriptomics identifies a signaling network coordinating endoderm and mesoderm diversification during foregut organogenesis. *Nat. Commun.* **2020**, *11*, 4158. [CrossRef] [PubMed]
8. Milano, F.; van Baal, J.W.; Buttar, N.S.; Rygiel, A.M.; de Kort, F.; DeMars, C.J.; Rosmolen, W.D.; Bergman, J.J.; van Marle, J.; Wang, K.K.; et al. Bone morphogenetic protein 4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells. *Gastroenterology* **2007**, *132*, 2412–2421. [CrossRef]

9.   Wang, D.H.; Clemons, N.J.; Miyashita, T.; Dupuy, A.J.; Zhang, W.; Szczepny, A.; Corcoran-Schwartz, I.M.; Wilburn, D.L.; Montgomery, E.A.; Wang, J.S.; et al. Aberrant epithelial-mesenchymal Hedgehog signaling characterizes Barrett's metaplasia. *Gastroenterology* **2010**, *138*, 1810–1822. [CrossRef]

10. Chang, C.L.; Lao-Sirieix, P.; Save, V.; De La Cueva Mendez, G.; Laskey, R.; Fitzgerald, R.C. Retinoic acid-induced glandular differentiation of the oesophagus. *Gut* **2007**, *56*, 906–917. [CrossRef]

11. Gharahkhani, P.; Fitzgerald, R.C.; Vaughan, T.L.; Palles, C.; Gockel, I.; Tomlinson, I.; Buas, M.F.; May, A.; Gerges, C.; Anders, M.; et al. Genome-wide association studies in oesophageal adenocarcinoma and Barrett's oesophagus: A large-scale meta-analysis. *Lancet Oncol.* **2016**, *17*, 1363–1373. [CrossRef]

12. Krishnan, U.; Mousa, H.; Dall'Oglio, L.; Homaira, N.; Rosen, R.; Faure, C.; Gottrand, F. Espghan-Naspghan Guidelines for the Evaluation and Treatment of Gastrointestinal and Nutritional Complications in Children with Esophageal Atresia-Tracheoesophageal Fistula. *J. Pediatric Gastroenterol. Nutr.* **2016**, *63*, 550–570. [CrossRef] [PubMed]

13. Koivusalo, A.I.; Pakarinen, M.P.; Lindahl, H.G.; Rintala, R.J. Endoscopic Surveillance after Repair of Oesophageal Atresia: Longitudinal Study in 209 Patients. *J. Pediatric Gastroenterol. Nutr.* **2016**, *62*, 562–566. [CrossRef] [PubMed]

14. Kato, T.; Noma, K.; Ohara, T.; Kashima, H.; Katsura, Y.; Sato, H.; Komoto, S.; Katsube, R.; Ninomiya, T.; Tazawa, H.; et al. Cancer-Associated Fibroblasts Affect Intratumoral CD8(+) and FoxP3(+) T Cells Via IL6 in the Tumor Microenvironment. *Clin. Cancer Res.* **2018**, *24*, 4820–4833. [CrossRef]

15. Wang, J.; Zhang, G.; Wang, J.; Wang, L.; Huang, X.; Cheng, Y. The role of cancer-associated fibroblasts in esophageal cancer. *J. Transl. Med.* **2016**, *14*, 30. [CrossRef]

16. Okawa, T.; Michaylira, C.Z.; Kalabis, J.; Stairs, D.B.; Nakagawa, H.; Andl, C.D.; Johnstone, C.N.; Klein-Szanto, A.J.; El-Deiry, W.S.; Cukierman, E.; et al. The functional interplay between EGFR overexpression, hTERT activation, and p53 mutation in esophageal epithelial cells with activation of stromal fibroblasts induces tumor development, invasion, and differentiation. *Genes Dev.* **2007**, *21*, 2788–2803. [CrossRef]

17. Mahlapuu, M.; Enerback, S.; Carlsson, P. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. *Development* **2001**, *128*, 2397–2406. [CrossRef]

18. Woo, J.; Miletich, I.; Kim, B.M.; Sharpe, P.T.; Shivdasani, R.A. Barx1-mediated inhibition of Wnt signaling in the mouse thoracic foregut controls tracheo-esophageal septation and epithelial differentiation. *PLoS ONE* **2011**, *6*, e22493. [CrossRef] [PubMed]

19. Shu, W.; Lu, M.M.; Zhang, Y.; Tucker, P.W.; Zhou, D.; Morrisey, E.E. Foxp2 and Foxp1 cooperatively regulate lung and esophagus development. *Development* **2007**, *134*, 1991–2000. [CrossRef] [PubMed]

20. Banham, A.H.; Beasley, N.; Campo, E.; Fernandez, P.L.; Fidler, C.; Gatter, K.; Jones, M.; Mason, D.Y.; Prime, J.E.; Trougouboff, P.; et al. The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. *Cancer Res.* **2001**, *61*, 8820–8829.

21. Shoemaker, M.B.; Husser, D.; Roselli, C.; Al Jazairi, M.; Chrispin, J.; Kuhne, M.; Neumann, B.; Knight, S.; Sun, H.; Mohanty, S.; et al. Genetic Susceptibility for Atrial Fibrillation in Patients Undergoing Atrial Fibrillation Ablation. *Circ. Arrhythm. Electrophysiol.* **2020**, *13*, e007676. [CrossRef]

22. Wang, Z.; Dolle, P.; Cardoso, W.V.; Niederreither, K. Retinoic acid regulates morphogenesis and patterning of posterior foregut derivatives. *Dev. Biol.* **2006**, *297*, 433–445. [CrossRef]

23. Chen, F.; Desai, T.J.; Qian, J.; Niederreither, K.; Lu, J.; Cardoso, W.V. Inhibition of Tgf beta signaling by endogenous retinoic acid is essential for primary lung bud induction. *Development* **2007**, *134*, 2969–2979. [CrossRef]

24. Lind, A.; Siersema, P.D.; Kusters, J.G.; Konijn, T.; Mebius, R.E.; Koenderman, L. The Microenvironment in Barrett's Esophagus Tissue Is Characterized by High FOXP3 and RALDH2 Levels. *Front. Immunol.* **2018**, *9*, 1375. [CrossRef] [PubMed]

25. Domyan, E.T.; Ferretti, E.; Throckmorton, K.; Mishina, Y.; Nicolis, S.K.; Sun, X. Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. *Development* **2011**, *138*, 971–981. [CrossRef] [PubMed]

26. Castillo, D.; Puig, S.; Iglesias, M.; Seoane, A.; de Bolos, C.; Munitiz, V.; Parrilla, P.; Comerma, L.; Poulsom, R.; Krishnadath, K.K.; et al. Activation of the BMP4 pathway and early expression of CDX2 characterize non-specialized columnar metaplasia in a human model of Barrett's esophagus. *J. Gastrointest. Surg.* **2012**, *16*, 227–237. [CrossRef] [PubMed]

27. Greten, F.R.; Grivennikov, S.I. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* **2019**, *51*, 27–41. [CrossRef]

28. Kaur, B.S.; Triadafilopoulos, G. Acid- and bile-induced PGE(2) release and hyperproliferation in Barrett's esophagus are COX-2 and PKC-epsilon dependent. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2002**, *283*, G327–G334. [CrossRef]

29. Souza, R.F.; Shewmake, K.; Terada, L.S.; Spechler, S.J. Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. *Gastroenterology* **2002**, *122*, 299–307. [CrossRef]

30. Abdel-Latif, M.M.; O'Riordan, J.; Windle, H.J.; Carton, E.; Ravi, N.; Kelleher, D.; Reynolds, J.V. NF-kappaB activation in esophageal adenocarcinoma: Relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Ann. Surg.* **2004**, *239*, 491–500. [CrossRef]

31. Morgan, C.; Alazawi, W.; Sirieix, P.; Freeman, T.; Coleman, N.; Fitzgerald, R. In vitro acid exposure has a differential effect on apoptotic and proliferative pathways in a Barrett's adenocarcinoma cell line. *Am. J. Gastroenterol.* **2004**, *99*, 218–224. [CrossRef]

32. Quante, M.; Bhagat, G.; Abrams, J.A.; Marache, F.; Good, P.; Lee, M.D.; Lee, Y.; Friedman, R.; Asfaha, S.; Dubeykovskaya, Z.; et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. *Cancer Cell* **2012**, *21*, 36–51. [CrossRef]

33. El-Serag, H.B.; Aguirre, T.V.; Davis, S.; Kuebeler, M.; Bhattacharyya, A.; Sampliner, R.E. Proton pump inhibitors are associated with reduced incidence of dysplasia in Barrett's esophagus. *Am. J. Gastroenterol.* **2004**, *99*, 1877–1883. [CrossRef]

34. Kastelein, F.; Spaander, M.C.; Biermann, K.; Steyerberg, E.W.; Kuipers, E.J.; Bruno, M.J. Nonsteroidal anti-inflammatory drugs and statins have chemopreventative effects in patients with Barrett's esophagus. *Gastroenterology* **2011**, *141*, 2000–2008; quiz e2013–e2014. [CrossRef]

35. Jankowski, J.A.Z.; de Caestecker, J.; Love, S.B.; Reilly, G.; Watson, P.; Sanders, S.; Ang, Y.; Morris, D.; Bhandari, P.; Brooks, C.; et al. Esomeprazole and aspirin in Barrett's oesophagus (AspECT): A randomised factorial trial. *Lancet* **2018**, *392*, 400–408. [CrossRef]

36. Dvorak, K.; Payne, C.M.; Chavarria, M.; Ramsey, L.; Dvorakova, B.; Bernstein, H.; Holubec, H.; Sampliner, R.E.; Guy, N.; Condon, A.; et al. Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: Relevance to the pathogenesis of Barrett's oesophagus. *Gut* **2007**, *56*, 763–771. [CrossRef] [PubMed]

37. De Gottardi, A.; Hadengue, A.; Dumonceau, J.M. Retinoids, bile acids and PPARs in Barrett's oesophagus. *Gut* **2008**, *57*, 137. [CrossRef]

38. Lam-Himlin, D.M.; Daniels, J.A.; Gayyed, M.F.; Dong, J.; Maitra, A.; Pan, D.; Montgomery, E.A.; Anders, R.A. The hippo pathway in human upper gastrointestinal dysplasia and carcinoma: A novel oncogenic pathway. *Int. J. Gastrointest. Cancer* **2006**, *37*, 103–109. [CrossRef] [PubMed]

39. Zhao, B.; Wei, X.; Li, W.; Udan, R.S.; Yang, Q.; Kim, J.; Xie, J.; Ikenoue, T.; Yu, J.; Li, L.; et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* **2007**, *21*, 2747–2761. [CrossRef]

40. Dietrich, C.; Kaina, B. The aryl hydrocarbon receptor (AhR) in the regulation of cell-cell contact and tumor growth. *Carcinogenesis* **2010**, *31*, 1319–1328. [CrossRef]

41. Zhao, B.; Li, L.; Wang, L.; Wang, C.Y.; Yu, J.; Guan, K.L. Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes Dev.* **2012**, *26*, 54–68. [CrossRef]

42. Koivusalo, A.I.; Rintala, R.J.; Pakarinen, M.P. Outcomes of fundoplication in oesophageal atresia associated gastrooesophageal reflux disease. *J. Pediatric Surg.* **2018**, *53*, 230–233. [CrossRef] [PubMed]

43. Van Wijk, M.; Knuppe, F.; Omari, T.; de Jong, J.; Benninga, M. Evaluation of gastroesophageal function and mechanisms underlying gastroesophageal reflux in infants and adults born with esophageal atresia. *J. Pediatric Surg.* **2013**, *48*, 2496–2505. [CrossRef] [PubMed]

44. Tugay, M.; Yildiz, F.; Utkan, T.; Ulak, G.; Gacar, N.; Erden, F. Impaired esophageal reactivity in adriamycin-induced rat esophageal atresia: An in vitro study. *J. Pediatric Surg.* **2001**, *36*, 1569–1573. [CrossRef] [PubMed]

45. Kauer, W.K.; Peters, J.H.; DeMeester, T.R.; Feussner, H.; Ireland, A.P.; Stein, H.J.; Siewert, R.J. Composition and concentration of bile acid reflux into the esophagus of patients with gastroesophageal reflux disease. *Surgery* **1997**, *122*, 874–881. [CrossRef]

46. Zaninotto, G.; Di Mario, F.; Costantini, M.; Baffa, R.; Germana, B.; Dal Santo, P.L.; Rugge, M.; Bolzan, M.; Naccarato, R.; Ancona, E. Oesophagitis and pH of refluxate: An experimental and clinical study. *Br. J. Surg.* **1992**, *79*, 161–164. [CrossRef]

47. Leedham, S.J.; Preston, S.L.; McDonald, S.A.; Elia, G.; Bhandari, P.; Poller, D.; Harrison, R.; Novelli, M.R.; Jankowski, J.A.; Wright, N.A. Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. *Gut* **2008**, *57*, 1041–1048. [CrossRef]

48. Martinez, P.; Mallo, D.; Paulson, T.G.; Li, X.; Sanchez, C.A.; Reid, B.J.; Graham, T.A.; Kuhner, M.K.; Maley, C.C. Evolution of Barrett's esophagus through space and time at single-crypt and whole-biopsy levels. *Nat. Commun.* **2018**, *9*, 794. [CrossRef]

49. Jego, M.; Volant, A.; Faycal, J.; Doucet, L.; Andlauer, E.; Delalande, A.H.; Cholet, F.; Nousbaum, J.B.; Gouerou, H.; Robaszkiewicz, M. Prevalence and topography of intestinal metaplasia in columnar lined esophagus. *Gastroenterol. Clin. Biol.* **2007**, *31*, 601–606. [CrossRef]

50. Yamamoto, Y.; Wang, X.; Bertrand, D.; Kern, F.; Zhang, T.; Duleba, M.; Srivastava, S.; Khor, C.C.; Hu, Y.; Wilson, L.H.; et al. Mutational spectrum of Barrett's stem cells suggests paths to initiation of a precancerous lesion. *Nat. Commun.* **2016**, *7*, 10380. [CrossRef]

51. Mourikis, T.P.; Benedetti, L.; Foxall, E.; Temelkovski, D.; Nulsen, J.; Perner, J.; Cereda, M.; Lagergren, J.; Howell, M.; Yau, C.; et al. Patient-specific cancer genes contribute to recurrently perturbed pathways and establish therapeutic vulnerabilities in esophageal adenocarcinoma. *Nat. Commun.* **2019**, *10*, 3101. [CrossRef] [PubMed]

52. Zhong, G.; Ahimaz, P.; Edwards, N.A.; Hagen, J.J.; Faure, C.; Kingma, P.; Middlesworth, W.; Khlevner, J.; Fiky, M.E.; Schindel, D.; et al. Identification and validation of novel candidate risk genes in endocytic vesicular trafficking associated with esophageal atresia and tracheoesophageal fistulas. *medRxiv* **2021**. [CrossRef]

53. Brosens, E.; Marsch, F.; de Jong, E.M.; Zaveri, H.P.; Hilger, A.C.; Choinitzki, V.G.; Holscher, A.; Hoffmann, P.; Herms, S.; Boemers, T.M.; et al. Copy number variations in 375 patients with oesophageal atresia and/or tracheoesophageal fistula. *Eur. J. Hum. Genet.* **2016**, *24*, 1715–1723. [CrossRef] [PubMed]

54. Iftikhar, S.Y.; James, P.D.; Steele, R.J.; Hardcastle, J.D.; Atkinson, M. Length of Barrett's oesophagus: An important factor in the development of dysplasia and adenocarcinoma. *Gut* **1992**, *33*, 1155–1158. [CrossRef] [PubMed]

55. Palles, C.; Chegwidden, L.; Li, X.; Findlay, J.M.; Farnham, G.; Castro Giner, F.; Peppelenbosch, M.P.; Kovac, M.; Adams, C.L.; Prenen, H.; et al. Polymorphisms near tbx5 and gdf7 are associated with increased risk for barrett's esophagus. *Gastroenterology* **2015**, *148*, 367–378. [CrossRef] [PubMed]

56. Levine, D.M.; Ek, W.E.; Zhang, R.; Liu, X.; Onstad, L.; Sather, C.; Lao-Sirieix, P.; Gammon, M.D.; Corley, D.A.; Shaheen, N.J.; et al. A genome-wide association study identifies new susceptibility loci for esophageal adenocarcinoma and barrett's esophagus. *Nat. Genet.* **2013**, *45*, 1487–1493. [CrossRef]

57. Casson, A.G.; Zheng, Z.; Evans, S.C.; Geldenhuys, L.; van Zanten, S.V.; Veugelers, P.J.; Porter, G.A.; Guernsey, D.L. Cyclin d1 polymorphism (g870a) and risk for esophageal adenocarcinoma. *Cancer* **2005**, *104*, 730–739. [CrossRef]

58. Ren, D.; Zheng, G.; Bream, S.; Tevebaugh, W.; Shaheen, N.J.; Chen, X. Single nucleotide polymorphisms of caudal type homeobox 1 and 2 are associated with barrett's esophagus. *Dig. Dis. Sci.* **2014**, *59*, 57–63. [CrossRef]

59. Su, Z.; Gay, L.J.; Strange, A.; Palles, C.; Band, G.; Whiteman, D.C.; Lescai, F.; Langford, C.; Nanji, M.; Edkins, S.; et al. Common variants at the mhc locus and at chromosome 16q24.1 predispose to barrett's esophagus. *Nat. Genet.* **2012**, *44*, 1131–1136. [CrossRef]

60. Becker, J.; May, A.; Gerges, C.; Anders, M.; Veits, L.; Weise, K.; Czamara, D.; Lyros, O.; Manner, H.; Terheggen, G.; et al. Supportive evidence for foxp1, barx1, and foxf1 as genetic risk loci for the development of esophageal adenocarcinoma. *Cancer Med.* **2015**, *4*, 1700–1704. [CrossRef]

61. Kala, Z.; Dolina, J.; Marek, F.; Izakovicova Holla, L. Polymorphisms of glutathione s-transferase m1, t1 and p1 in patients with reflux esophagitis and barrett's esophagus. *J. Hum. Genet.* **2007**, *52*, 527–534. [CrossRef] [PubMed]

62. Bull, L.M.; White, D.L.; Bray, M.; Nurgalieva, Z.; El-Serag, H.B. Phase i and ii enzyme polymorphisms as risk factors for barrett's esophagus and esophageal adenocarcinoma: A systematic review and meta-analysis. *Dis. Esophagus* **2009**, *22*, 571–587. [CrossRef] [PubMed]

63. McElholm, A.R.; McKnight, A.J.; Patterson, C.C.; Johnston, B.T.; Hardie, L.J.; Murray, L.J.; Finbar, G. A population-based study of igf axis polymorphisms and the esophageal inflammation, metaplasia, adenocarcinoma sequence. *Gastroenterology* **2010**, *139*, 204–212.e3. [CrossRef]

64. Moons, L.M.; Kusters, J.G.; van Delft, J.H.; Kuipers, E.J.; Gottschalk, R.; Geldof, H.; Bode, W.A.; Stoof, J.; van Vliet, A.H.; Ketelslegers, H.B.; et al. A pro-inflammatory genotype predisposes to barrett's esophagus. *Carcinogenesis* **2008**, *29*, 926–931. [CrossRef] [PubMed]

65. Buas, M.F.; He, Q.; Johnson, L.G.; Onstad, L.; Levine, D.M.; Thrift, A.P.; Gharahkhani, P.; Palles, C.; Lagergren, J.; Fitzgerald, R.C.; et al. Germline variation in inflammation-related pathways and risk of barrett's oesophagus and oesophageal adenocarcinoma. *Gut* **2017**, *66*, 1739–1747. [CrossRef]

66. Yan, C.; Ji, Y.; Huang, T.; Yu, F.; Gao, Y.; Gu, Y.; Qi, Q.; Du, J.; Dai, J.; Ma, H.; et al. An esophageal adenocarcinoma susceptibility locus at 9q22 also confers risk to esophageal squamous cell carcinoma by regulating the function of barx1. *Cancer Lett.* **2018**, *421*, 103–111. [CrossRef]

67. Abnet, C.C.; Freedman, N.D.; Hu, N.; Wang, Z.; Yu, K.; Shu, X.O.; Yuan, J.M.; Zheng, W.; Dawsey, S.M.; Dong, L.M.; et al. A shared susceptibility locus in plce1 at 10q23 for gastric adenocarcinoma and esophageal squamous cell carcinoma. *Nat. Genet.* **2010**, *42*, 764–767. [CrossRef]

68. Dura, P.; van Veen, E.M.; Salomon, J.; te Morsche, R.H.; Roelofs, H.M.; Kristinsson, J.O.; Wobbes, T.; Witteman, B.J.; Tan, A.C.; Drenth, J.P.; et al. Barrett associated mhc and foxf1 variants also increase esophageal carcinoma risk. *Int. J. Cancer* **2013**, *133*, 1751–1755. [CrossRef]

69. Wideroff, L.; Vaughan, T.L.; Farin, F.M.; Gammon, M.D.; Risch, H.; Stanford, J.L.; Chow, W.H. Gst, nat1, cyp1a1 polymorphisms and risk of esophageal and gastric adenocarcinomas. *Cancer Detect. Prev.* **2007**, *31*, 233–236. [CrossRef]

70. Gross, R.E. *The Surgery of Infancy and Childhood: Its Principles and Techniques*; W.B. Saunders Co.: Philadelphia, PA, USA, 1953.

71. Lundell, L.R.; Dent, J.; Bennett, J.R.; Blum, A.L.; Armstrong, D.; Galmiche, J.P.; Johnson, F.; Hongo, M.; Richter, J.E.; Spechler, S.J.; et al. Endoscopic assessment of oesophagitis: Clinical and functional correlates and further validation of the los angeles classification. *Gut* **1999**, *45*, 172–180. [CrossRef]

72. Rasanen, K.; Itkonen, O.; Koistinen, H.; Stenman, U.H. Emerging roles of spink1 in cancer. *Clin. Chem.* **2016**, *62*, 449–457. [CrossRef] [PubMed]

73. Jennings, R.E.; Berry, A.A.; Kirkwood-Wilson, R.; Roberts, N.A.; Hearn, T.; Salisbury, R.J.; Blaylock, J.; Piper Hanley, K.; Hanley, N.A. Development of the human pancreas from foregut to endocrine commitment. *Diabetes* **2013**, *62*, 3514–3522. [CrossRef] [PubMed]

74. Gene Expression for Spink1 (ensg00000164266.10). Available online: https://gtexportal.org/home/gene/SPINK1 (accessed on 15 October 2021).

75. Alexander, D.H.; Lange, K. Enhancements to the admixture algorithm for individual ancestry estimation. *BMC Bioinform.* **2011**, *12*, 246. [CrossRef] [PubMed]

76. Wray, N.R.; Goddard, M.E.; Visscher, P.M. Prediction of individual genetic risk to disease from genome-wide association studies. *Genome Res.* **2007**, *17*, 1520–1528. [CrossRef]

77. Clc Genomics Workbench User Manual. Available online: https://digitalinsights.qiagen.com/technical-support/manuals/ (accessed on 15 October 2021).

78. Bus, P.; Siersema, P.D.; Verbeek, R.E.; van Baal, J.W. Upregulation of mirna-143, -145, -192, and -194 in esophageal epithelial cells upon acidic bile salt stimulation. *Dis. Esophagus* **2014**, *27*, 591–600. [CrossRef] [PubMed]