# Epithelial and subepithelial players in chronic intestinal disease

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# Chapter 1

## Introduction and aim of the thesis

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## 1.1. Introduction

The human gut is composed of two main segments, the small intestine and the large intestine, that forms a continuous tract for food digestion and absorption. The length of the intestine is dependent on age and height. The small intestine is approximately 3-5 meters long in adults and subdivided into the duodenum, jejunum and ileum [1]. The large intestine is about 1.5 meters long and is subdivided into the cecum, colon, rectum, and anal canal [2]. The main function of the gut is digestion and absorption of food and fluids and at the same time form a tight barrier for potential pathogens. The small intestine contains large amounts of potential aggressive digestive enzymes necessary for chemical digestion and is equipped by a mucosa specialized in absorption [3]. The intestine absorbs water and nutrients from the food into the bloodstream by either diffusion or active transport. Bacteria in the colon help to ferment undigested components so that humans, as omnivores, can utilize food from both animal and plant sources to provide nutrition and energy for the body [4].

Maintaining intestinal integrity of the intestinal epithelium, where most physiological digestive, absorptive and secretory processes take place, is crucial [5]. The intestinal epithelium is a single layer of cells that is completely renewed every 4-5 days. This remarkably rapid epithelial cell renewal is driven by $LGR5^+$ multipotent intestinal stem cells (ISCs) located at the bottom of the epithelial crypts [5]. ISCs continuously proliferate and differentiate into specialized cells that serve multiple functions [6,7]. **Figure 1** shows schematically the distribution of the main classes of intestinal epithelial cells in the crypt-villus axis. Enterocytes make up most (~80%) of the intestinal epithelium and specialized in absorbing nutrients [5]. Goblet cells secrete mucins, particularly mucin 2, that create a protective mucus layer on top of the intestinal epithelium [8]. Relative numbers of goblet cells in the epithelium increase along the intestinal tract from duodenum (~4%) to the descending colon (~16%). Enteroendocrine cells sense the luminal content of the gut, in particular nutrients, and in response secrete a great variety of different hormones. They constitute a heterogeneous group of cells with around 15 subtypes characterized, based on cellular

morphology, specific hormones produced and subtype-specific markers [9]. Approximately 1% of the differentiated intestinal epithelial cells in the human gut are enteroendocrine cells. Paneth cells produce lysozymes, antimicrobials and defensins and play a key role in the innate immunity [10]. The epithelial cells moving up from the crypt to the villus are closely connected by semipermeable cellular junctions in order to prevent leakage of transported solutes and water and selectively seal the paracellular pathway for harmful substances [6,11,12].

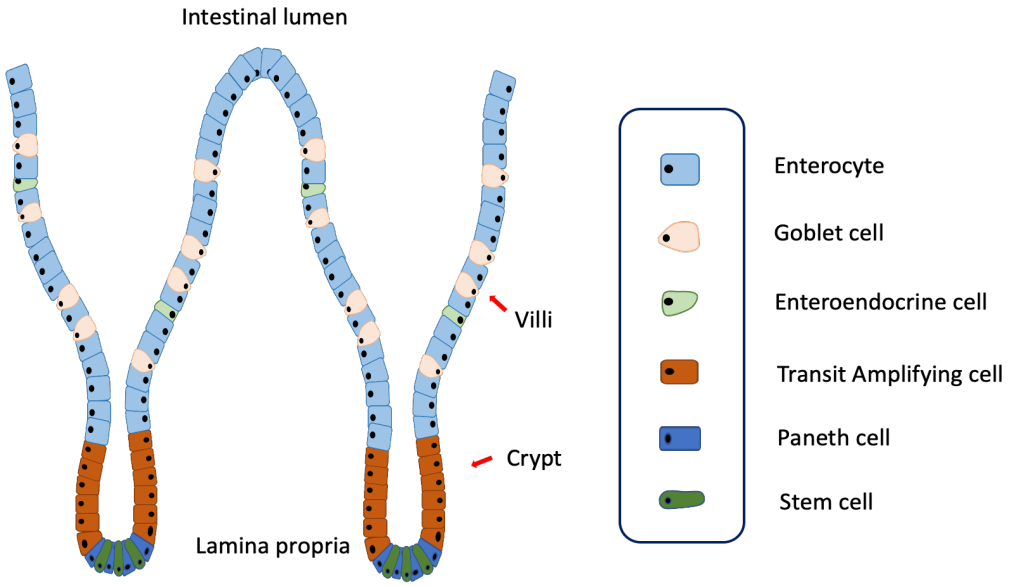


**Figure 1:** Major cell types and structural representation of human intestinal epithelium.

Beneath the epithelial layer is the lamina propria. The lamina propria is a thin layer of connective tissue rich in cells, such as fibroblasts, myofibroblasts, endothelial cells, lymphocytes, macrophages, dendritic cells, mast cells and leukocytes [13]. The immune system in the intestine is tolerant towards the normal intestinal microbiota and harmless food antigens, however, a guardian against pathogenic microbiota and harmful substances [14,15]. For this reason, the lamina propria contains many immune cells that control the intestinal mucosal immune response [14,16,17]. Fibroblasts and myofibroblasts in the lamina propria are important contributors to inflammation and wound healing processes. In inflammatory conditions, macrophages and mast cells release fibroblast growth factors, such as PDGF and TGF-β, to activate fibroblasts and

to promote their proliferation and migration to sites of injury [18]. Activated myofibroblasts release themselves from surrounding cells and extra cellular matrix (ECM), move towards the wound site and produce a collagen matrix to initiate tissue regeneration [19]. However, in chronic intestinal disease, fibroblasts produce excessive ECM leading to intestinal fibrosis.

Among all the harmful external factors to which we are currently exposed, cigarette smoking is believed to be one of the largest preventable risk factors causing intestinal diseases, such as peptic ulcers, inflammatory bowel diseases (IBD) and colon cancer [20]. Cigarette smoke has many pleiotropic effects, influencing mucus production, microbiota composition, mucosal integrity and mucosal immune responses [20]. Cigarette smoking increases the risk for colon cancer by influencing epithelial cell cycle-related proteins and epithelial mesenchymal transition (EMT) [21]. However, only little is known about the direct effect of cigarette smoke on intestinal epithelial regeneration and morphogenesis in humans. *In vitro* models have been adopted from research with lung cells that were exposed to standardized culture media saturated with cigarette smoke [22,23]. Colon cancer-derived Caco-2 epithelial cells have been exposed to such cigarette smoke extract (CSE), which resulted in reduced cellular metabolic activity and impaired claudin-1 and E-cadherin expression, key factors in maintain epithelial barrier integrity [24]. These studies were performed with Caco-2 cells in two-dimensional monolayers [24]. Recent developments in creating 3-dimensionally (3D)-grown human intestinal organoids from adult stem cells residing in the crypts [25-28] now allows a much more comprehensive analysis of environmental factors on the human intestinal epithelium. Intestinal organoids are grown in conditions that foster the stem cells and can be maintained in culture for months to years. Intestinal organoids are genetically stable during long term-culture and growth conditions can be adapted to allow terminal differentiation to the more mature epithelium enriched for enterocytes, goblet cells, Paneth cells and enteroendocrine cells [29]. Interestingly, differentiated organoids resemble the tissue that they originate from, enriched for location (duodenum, jejunum, ileum or colon)-specific markers [30]. Thus, intestinal organoids are composed of polarized epithelial layers

surrounding functional lumens in which all intestinal cell types are preserved and are excellent models to study the direct effect of cigarette smoke on human intestinal epithelial morphogenesis and function.

Inflammatory bowel disease (IBD) is characterized by chronic and relapsing inflammation of the gastrointestinal tract. Two main forms can be distinguished; Crohn's disease (CD) and ulcerative colitis (UC). The highest prevalence is reported in Europe and North America, ranging from 400-500/100,000 persons [31], and is still increasing. Moreover, IBD is increasingly diagnosed in countries adopting a Western life style [32,33]. CD is characterized by patchy transmural inflammation that may occur anywhere in the gastrointestinal track, but typically involves the ileum and colon [34]. UC, in contrast, is limited to colon and presents as continuous mucosal inflammation starting at the rectum and spreads up into the colon [35]. The whole colon is inflamed, e.g. pan-colitis, in the severest form of UC [36]. CD and UC are multifactorial diseases that are caused by an unfortunate interplay between genetic and environmental factors that lead to an uncontrolled inflammatory response directed against gut bacteria [37]. Over 240 genetic loci have been identified predispose for IBD, some specific for CD, some for UC and many associated with both forms [38,39]. The genetic make-up of an individual makes him/her more or less susceptible to external environmental factors, such as cigarette smoking, high-fat/ low-fiber diet and social stress, that are critical components in the etiology of IBD [40]. Most patients are diagnosed with IBD in early adulthood (aged 20-30) and required life-long disease management as there is no cure. Drugs like corticosteroids, mesalazine, immunomodulators (thiopurines, methotrexate and tofacitinib) and biologicals (anti-TNF-alpha antibodies, vedolizumab, ustekinumab) are used to induce and maintain disease remission [41,42]. However, many patients show primary-nonresponse or loss of response to these drugs.

Interestingly, cigarette smoking has a remarkable opposite effect in CD and UC, as it increases the risk of CD and decreases the risk of UC. Moreover, smoking increases the risk of intestinal fibrosis, a common and serious complication of CD [23, 24]. There also

appears to be a location-specific effect, since smoking patients with ileal CD in particular show more inflammation and fibrostenotic disease than non-smoking patients and requires dilatation or surgical resection [43]. Cigarette smoke decreases the anti-inflammatory cytokine IL-10, increases pro-inflammatory chemokines and cytokines such as CCR6, CCL 20, IL-6 and IL-8, activates dendritic cells, and increases the recruitment of CD4+, CD8+ T cells and CD11b+ dendritic cells especially in the ileum [20,44].

The beneficial effects of smoking in UC are thought to be mediated by specific components in smoke, such as carbon monoxide and nicotine. Carbon monoxide could modify the inflammatory response by inhibition neutrophil extracellular trap formation (netosis) by neutrophils, a prominent cell type in the inflammatory profile of UC [45]. Nicotine reduces the production of pro- inflammatory cytokines by activating alpha-7 nicotinic receptor in immune cells, such as macrophages and dendritic cells [46]. These anti-inflammatory effects of cigarette smoke have to be balanced against the potential carcinogenic effect on intestinal epithelial cells.

It is generally believed that intestinal inflammation also triggers the fibrotic response, but intestinal fibrogenesis can become independent of inflammation in later stages of disease [47]. An interesting protein with respect to smoking, inflammation and fibrogenesis is vascular adhesion protein-1/semicarbazide-sensitive amine oxidase (VAP-1/SSAO, encoded by the *AOC3* gene). VAP-1 is a transmembrane protein abundantly expressed by vascular endothelial cells, smooth muscle cells, pericytes, adipocytes and subepithelial myofibroblasts [48]. VAP-1 is a multifunctional protein that acts both as a receptor that supports leukocyte extravasation, as well as an enzyme that oxidates primary amines and produces hydrogen peroxide [49]. Both activities promote tissue inflammation and fibrosis, and VAP-1 expression is typically enhanced in inflamed tissues [49,50]. Interestingly, soluble VAP-1 levels are increased in individuals who smoke compared to non-smokers [51]. It is, however, unknown whether cigarette smoke actually affects tissue expression of VAP-1 and thereby promote could intestinal inflammation and fibrosis.

Under persistent epithelial inflammatory condition, myofibroblasts secrete and deposit abnormal amounts of extra cellular matrix (ECM) proteins that promotes the development of intestinal stenosis [52]. Although important progress has been made in controlling inflammation in IBD, this does not necessarily also prevent, or even slow down, intestinal fibrogenesis. So far, no drug-based therapies are available to suppress and/or reverse intestinal fibrosis. Therefore, a better understanding of the specific molecular signaling pathways involved in intestinal fibrosis is therefore needed to be able to develop effective treatment strategies [53]. An alternative approach is to hitch-hike on progress made in drug development for the treatment of fibrosis in other organs and/or diseases and test such drugs for the treatment of intestinal fibrosis. For example, pirfenidone has been shown to be an efficacious anti-fibrotic compound in idiopathic pulmonary fibrosis, a devastating, progressive fibrotic lung disease with a median survival of 3–5 years. It is a well-tolerated compound that decreases the rate of decline in forced vital capacity and increases the progression-free survival (PFS) time and overall survival (OS) time [54-56]. Pirfenidone has therapeutic value in the early phase of disease, but also in more advanced and even in perioperative IPF patients [57,58]. Various cellular and molecular mechanisms have been characterized that contribute to the anti-fibrotic action of pirfenidone in lung fibrosis. Pirfenidone inhibits lung fibroblast migration and ECM protein production, including collagen type I and III, fibronectin and tenascin-c [59]. Moreover, pirfenidone suppresses TGF-β1-induced gene expression of *COL1A1*, C*OL3A1* and *CHD2* and collagen protein production by blocking the activation of the Smad, p38MAPK and Akt signaling pathways [60,61]. TGF-β1 is known to be the master regulator of intestinal fibrosis promoting ECM protein synthesis and inhibiting ECM degradation [62]. Also, TGF-β1 is known to activate the mTORC1 signaling pathway, which is a central regulator of cell metabolism, proliferation, and protein synthesis. Important downstream execution proteins are 4E-BP1, a translation repressor protein, and p70S6K that targets the S6 ribosomal protein [63]. It will be interesting to determine whether drugs like pirfenidone may also suppress the fibrogenic phenotype of intestinal fibroblasts, and thereby can be potentially be used clinically to suppress intestinal fibrosis.

## 1.2. The aim of the thesis

The general aim of this thesis is to explore the effects of cigarette smoke on the human intestinal epithelium and subepithelial myofibroblasts, two intimately connected cell layers that form the border between the intestinal lumen and the underlying tissue, which in turn links to the rest of the human body. Our research primarily focused on its relation to intestinal carcinogenesis, inflammation and fibrosis, as well as identifying therapeutic targets for the treatment of chronic intestinal diseases.

In **Chapter 2**, we studied the effect of cigarette smoke on human intestinal epithelial cells by using colonic organoids and 3D-speroids of Caco-2 cells. We analyzed the direct effect and long-lasting effects of cigarette smoke on epithelial morphogenesis, regenerative capacity, polarity and barrier function in particular with relevance to the development of colon cancer.

In **Chapter 3,** we analyzed the relationship between smoking, mucosal VAP-1 expression and intestinal fibrosis in IBD patients. Moreover, and studied the direct effect of cigarette smoke on VAP-1 expression in primary human intestinal fibroblasts and intestinal organoids in order to establish a possible role of VAP-1 in intestinal inflammation and fibrosis.

In **Chapter 4**, we characterized a novel VAP-1-expressing cell type that we detected in the human intestinal epithelium and analyzed their presence in healthy jejunum, ileum and colon, as well as in inflamed and stenotic intestinal tissues of IBD patients.

In **Chapter 5**, we investigated the potential anti-fibrotic effects of pirfenidone on primary human intestinal fibroblasts (p-hIFs), including its effect on TGF-β1-mediated mTOR-p70S6K1 signaling.

In **Chapter 6**, we summarize the results described in this thesis and present future perspectives.

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