Regulation of antimicrobial peptides in the gastrointestinal tract

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ABBREVATIONS

APC	Adenomatous polyposis coli
AMP	Antimicrobial peptide
AMV	Avian Myeloblastosis Virus
BD	β-defensin
BT	Bacterial translocation
BMD	Bone mineral density
CCl_4	Carbon tetrachloride
CD	Crohn's disease
CRS-1C	Cryptdin-related sequence-1C
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
Gfp	Green fluorescent protein
GIT	Gastrointestinal tract
GSK-3	Glycogen synthase kinase-3
HBD-1/-2	Human β-defensin -1/-2
HD-5/-6	Human α-defensin -5/-6
HIP/PAP	Hepatocarcinoma–intestine–pancreas/pancreatic–associated protein 3
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
l	Liter
LC	Liver cirrhosis
LRP5/6	Low-density lipoprotein receptor related protein 5/6
LL-37	Cathelicidin LL-37
ml	Milliliter
MAMP	Microbe-associated molecular pattern
MDP	Muramyldipeptide
mDefa 21	C57BL6 strain specific analogue to cryptdin 4
mDefa 24	Mouse defensin 24
NF-κB	Nuclear factor 'kappa-light-chain-enhancer'
PAMP	Pathogen-associated molecular pattern
PVL	Portal vein ligation/prehepatic portal hypertension
PC	Paneth cell
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
rBD 1/2	Rat beta defensin 1/2
rCRAMP	Rat analogue to cathelicidin
rCrypt 5/7	Rat cryptdin 5/7
Reg3γ	Regenerating islet-derived protein 3 gamma
rHIP/PAP	Rat hepatocarcinoma–intestine–

	pancreas/pancreatic–associated protein 3
rNP3	Rat neutrophil protein 3
RT	Reverse transcriptase
rpm	Revolutions per minute
RT	Room temperature
SEM	Standard error of mean
sPLA2	Secreted Phospholipase 2
SNP	Single nucleotide polymorphism
TCF	T cell transcription factor
TGF-β	Transforming growth factor-β
TLR	Toll like receptor
UC	Ulcerative colitis

SUMMERY

The intestinal epithelium has an enormously large surface, which is constantly exposed to high amounts of pathogens and commensal bacteria. In order to prevent pathogens or other microorganisms from overcoming this barrier, good protection is needed. In the small intestine, human α-defensin 5 (HD-5) and 6 (HD-6) are important components of this barrier. Paneth cells, which are secretory epithelial cells, produce these antimicrobial peptides. The cells are located at the base of the crypt in the small intestine, where they play a central role in the microbial defense and regulating the composition of the intestinal microbiota. In ileal Crohn's disease, which belongs to the inflammatory bowel diseases, HD-5 and HD-6 levels are reduced, which leads to a changed composition of the intestinal flora and a poorer barrier function of the intestinal mucosa. Underlying mechanisms are genetically but also non-genetically influencing the Paneth cell function on different levels. The Wnt signaling pathway plays a key role in maintaining intestinal homeostasis. On the one hand it keeps the intestinal epithelial cells in a proliferating state, on the other hand it regulates the differentiation and maturation of Paneth cells. It is also instrumental in the regulation of HD-5 and -6. Former results from our group revealed a link between the decreased expression of HD-5 and -6 and the Wnt transcription factor TCF-4. Independent of inflammation, patients with ileal Crohn's disease show decreased TCF-4 expression levels. But not only TCF-4 is affected, our group could also show that the receptor LRP6, which is essential for the stabilization of β-catenin in the cytoplasm is affected. Studies showed a decreased expression in ileal Crohn's disease. The results of genetic investigations are also exciting; In the LRP6 gene, a polymorphism was identified that occurs significantly more frequently in a subgroup (patients> 18) than in healthy subjects.

In the present work the consequences of a disturbed Paneth cell function, but also potential causes were further investigated. Emphasis was placed on the patient side, but the microbial side of the balance was also examined using animal models. It could be shown that LRP6 directly regulates the expression of HD-5 and -6 whereas the expression of LRP5, as a co-expressed receptor of LRP6, is barely altered in patients and also *in vitro* there is no evidence for a direct regulation of HD-5 and -6.

It was possible to show the high heterogeneity of LRP5 in several populations studied and no genetic association in all subgroups of Crohn's disease was found. Studying TCF-1 expression revealed decreased levels in patients with ileal Crohn's

disease. Further results from a knockout mouse model showed that a TCF-1 knockout leads to a lower expression of cryptdines (mouse α-defensins). Furthermore, the role of antimicrobial peptides in the intestine could be further clarified in a rat liver cirrhosis model, since the transfer of commensal bacteria occurring here through the intestinal epithelium could be explained by a reduced expression of some antimicrobial peptides. Although many factors which play a role in the development of ileal Crohn's disease are still unknown, the present work has provided new insights into the mechanisms that lead to a well-balanced homeostasis at the epithelial site in the small intestine. Furthermore, the important role of the Wnt signaling pathway for the development of ileal Crohn's disease was underlined. Hopefully, these additional insights into the complex pathogenesis of the disease will one day provide new approaches to therapy.

ZUSAMMENFASSUNG

Das Epithel des Darmes besitzt eine enorm große Oberfläche, welche ständig erheblichen Mengen von Pathogenen und kommensalen Bakterien ausgesetzt ist. Um zu verhindern, dass Pathogene oder auch andere Mikroorganismen diese Barriere überwinden, ist ein guter Schutz nötig. Im Dünndarm sind die humanen α- Defensine 5 (HD-5) und 6 (HD-6) wichtige Komponenten dieser Barriere. Diese antimikrobiellen Peptide werden von Panethzellen produziert. Panethzellen sind sekretorischen Epithelzellen und befinden sich an der Basis der Krypte im Dünndarm. Dort spielen sie eine zentrale Rolle bei der mikrobiellen Abwehr und der Regulation der bakteriellen Zusammensetzung der Darmflora. Bei Morbus Crohn des Dünndarms, diese Erkrankung zählt zu den chronisch entzündlichen Darmerkrankungen, kommt es zu einer verminderten Expression von HD-5 und HD-6 und dadurch zu einer veränderten Zusammensetzung der Darmflora und zu einer schlechteren Barrierefunktion der Darmmukosa. Grundlage hierfür sind verschiedene genetische aber auch nicht- genetische Defekte, welche die Panethzellfunktion auf verschiedenen Ebenen beeinflussen.

Der Wnt Signalweg spielt eine zentrale Rolle, wenn es darum geht die Homöostase im Darm aufrecht zu erhalten. Er hält die intestinalen Epithelzellen einerseits in einem proliferierenden Zustand, andererseits reguliert er die Differenzierung und das Ausreifen der Panethzellen. Ebenso ist er an der Regulation von HD-5 und -6 maßgeblich beteiligt. Unsere Arbeitsgruppe konnte bereits zeigen, dass es eine

Verbindung zwischen der verminderten Expression von HD-5 und -6 und dem Wnt Transkriptionsfaktor TCF-4 gibt. Entzündungsunabhängig zeigen Patienten mit Morbus Crohn des Dünndarms eine verminderte TCF-4 Expression. Aber nicht nur TCF-4 ist betroffen, es konnte auch gezeigt werden, dass der Rezeptor LRP6 betroffen ist, welcher für die Stabilisierung von ß- Catenin im Cytoplasma essentiell ist. Untersuchungen zeigten eine verminderte Expression bei Morbus Crohn Patienten. Spannend sind auch die Ergebnisse genetischer Untersuchungen; im LRP6 Gen wurde ein Polymorphismus identifiziert, der in einer Untergruppe (Patienten >18) deutlich häufiger auftritt als in Gesunden.

In der hier vorgestellten Arbeit wurde an diese Ergebnisse angeknüpft und die Folgen einer gestörten Panethzellfunktion, aber auch die Faktoren die potentiell dazu führen, weiter untersucht. Hierbei wurde ein Schwerpunkt auf die Patientenseite gelegt, aber auch die mikrobielle Seite wurde mithilfe von Modellen untersucht. Es konnte gezeigt werden, dass LRP6 die Expression von HD-5 und -6 direkt reguliert während die Expression von LRP5, als ein Co-Rezeptor von LRP6, in Patienten kaum verändert ist und sich auch *in vitro* keine Hinweise auf eine direkte Regulation von HD-5 und -6 finden lassen. Ebenso zeigte sich, dass LRP5 sehr heterogen in mehreren untersuchten Populationen vorliegt und keine genetische Assoziation in allen untersuchten Subgruppen von Morbus Crohn vorliegt. Die Untersuchungen von TCF-1 zeigten eine erniedrigte Expression in Patienten mit Morbus Crohn des Dünndarms. Ergebnisse aus einem Knockout Mausmodell zeigten, dass ein TCF-1 Knockout zu einer niedrigeren Expression von Cryptdinen führt. Weiter konnte in einem Leberzirrhose- Tiermodel die Rolle antimikrobieller Substanzen weiter geklärt werden, da der hier auftretende Übertritt von kommensalen Bakterien durch das Darmepithel mit einer verminderten Expression einiger antimikrobieller Peptide erklärt werden konnte.

Obwohl immer noch viele Faktoren, die in der Entstehung von Morbus Crohn des Dünndarms eine Rolle spielen unbekannt sind, konnte die hier vorliegende Arbeit neue Einblicke in die Mechanismen liefern, die das Aufrechterhalten der Balance zwischen Bakterien und Mensch im Dünndarm ermöglichen. Weiter wurde die wichtige Rolle des Wnt Signalwegs für die Entstehung von Morbus Crohn des Dünndarms unterstrichen. Hoffentlich ermöglichen diese weiteren Einblicke in die komplexe Pathogenese der Erkrankung eines Tages neue Ansätze für eine Therapie

1. INTRODUCTION

1.1 The human gastrointestinal tract

The gastrointestinal tract (GIT) consists of different compartments (figure 1). The first part is the oral cavity followed by the pharynx, the oesophagus and the stomach, which can be subdivided into four sections. The last of these sections and the pylorus enables the emptying into the small intestine (Tortora and Derrickson 2008). These upper parts of the GIT mediate the ingestion and the digestion of food. The lower part is responsible for the absorption of nutrients and water. It consists of the small intestine (duodenum, jejunum and ileum) with an approximate length of 5 meters.

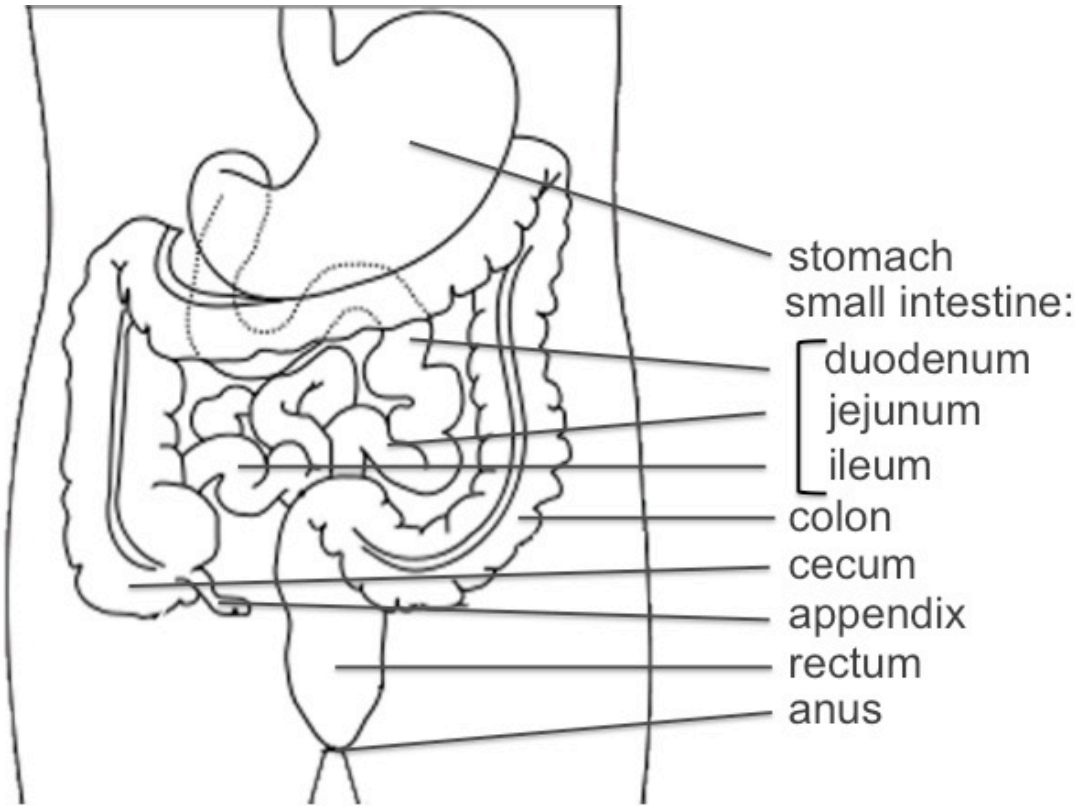

Figure 1: The gastrointestinal tract (adapted from William Crochet (science-et-vie.net))

Its surface is covered by finger-like structures, called villi, which increase the surface to ensure optimal nutrition uptake. Most of the digestion takes place in the small intestine, catalysed by enzymes produced in the pancreas and released into the small bowel (Clark 2005). The small intestine is followed by the large intestine; whereas the colon mediates absorption of water and electrolytes and then, finally, unneeded material is released through the rectum and the anus as stool (Tortora and

Derrickson 2008). The microscopically composition of the gut wall resembles each other in all sections of the gut. The outer layer, which is named serosa, consists of thin connective tissue. The subjacent layer is the submucosa followed by the mucosa, which is subdivided into three layers: The lamina muscularis mucosae, consisting of thin muscle tissue, which enables the gut to contract crosswise as well as lengthwise to convey the food bolus. A layer of connecting tissue, called the lamina propria, harbouring blood vessels and lymphatic nodules supplying the epithelium. The intestinal epithelium forms the innermost layer. It is responsible for most of the absorptive, digestive and secretory processes, but also has a critical role in maintaining an effective barrier against invading pathogens and in controlling the normal gut microbiota (Dorland n.d.; Tortora and Derrickson 2008, Lüllmann-Rauch 2009). This aspect will be discussed in the following section.

1.2 Immunity

1.2.1 Innate immunity and the microbiology of the human GIT

In humans, like in all mammals, the immune system is divided into a fast responding, simpler structured part, called the innate immune system and a more complex part with a slower but very specific response to pathogens, called the adaptive immune system. Whereas the adaptive immunity reacts to specific antigens, facilitates vaccinations and even recognises non-infectious structures, the innate immunity aims on a faster but more generic first line of defence (Janeway 2001). The intestinal epithelium is a constantly renewing self-regenerating tissue, with a renewal rate which exceeds all other tissues in the human body (Gregorieff and Clevers 2005). The enormous surface of the small intestinal epithelium with its crypts and villis is constantly challenged with high numbers of microorganisms comprising the healthy gut microbiota but also pathogens. The microbiota makes up to 1-2 kg, and consists of 10^{13} to 10^{14} microorganisms and outnumbers the amount of cells in the human body by far (Martín et al. 2014, Lee and Mazmanian 2010). The numbers of bacteria are rising with increasing distance from the stomach and therefore increasing pH. Because of the high amounts of bactericidal properties, gastric and bile acids, the stomach, and also the proximal small intestine are relatively depleted from bacteria in comparison to the ileum. The ileum is colonized by large amounts of anaerobic and aerobic bacteria. The colon however represents the most densely populated part of the GI-tract, harbouring 10^{12} bacteria/g of intestinal content (Sekirov et al. 2010). Due to this enormous bacterial load it is elementary to have a well-balanced

homeostasis at the epithelial site. The epithelium, which is the first line of defence against invading pathogens, confers an efficient mechanical barrier and restricts bacteria, fungi, yeasts and even viruses from invading the mucosa and preserves the homeostasis towards symbiotic microorganisms. Besides generating mechanical borders, epithelial cells maintain a chemical barrier system; including the low pH of the stomach acid, produced by parietal cells and the mucus which is secreted by goblet cells. Moreover there is a complex array of antimicrobial peptides and polypeptides as well as proteins with bactericidal properties produced by different cell types, mainly the Paneth cells in the small intestine but also all epithelial cells (Tollin et al. 2003; Howell et al. 2003). Germ-line encoded pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) located in the outer membrane, or intracellular NOD-like receptors (NLR), recognize specific highly conserved patterns, the so called “microbe associated molecular patterns” (MAMPs) on the microbial surface of the resident gut microbiota but also on pathogens (Litman, Cannon, and Dishaw 2005). As a consequence of PRR stimulation by their corresponding ligands, a pro-inflammatory response takes place which often occurs via activation of MyD88 and NF-κB or MAP-kinases (Takeda and Akira 2004). The rapid and generic first line of defence additionally leads to PRR signaling in immune cells in the lamina propria. This also enables an adequate inflammatory reaction in response to invading pathogens (Fukata and Arditi 2013; Tosi 2005). Low expression of the TLRs and their location at the basolateral side enables very low inflammation rates in the healthy gut despite the high bacterial load (Abreu 2010; Cario 2010).

But not only the number of bacteria is controlled, also their composition. The commensal microbiota consists of *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* (Nuding et al. 2009). In the ileum the bacterial composition mostly consists of aerobic and anaerobic bacteria like *Enterobacter* and *Lactobacilli* whereas predominately anaerobic phyla like *Bacteroides*, *Bifidobacteria*, *Fusobacteria*, *Clostridium* and *Peptostreptococci* provide most of the microbiota in the colon (Peterson et al. 2008). Antimicrobial peptides (AMPs) produced constitutively or inducible are abundant in a broad range. They are provided by almost all epithelial cells but, as mentioned, also by specialised cells. AMPs are not only produced by humans, but also found in every organism. In invertebrates they often are the major defence mechanism (Tincu and Taylor 2004). The human antimicrobial defence system has co-evolved with the microbes that have been challenging it. In our gut, this close exposure has led to a commensalism or in the ideal case to a mutualistic co-

existence (Neish 2009). To maintain a healthy balance between these microorganisms, the human immune system, on the one hand, has to tolerate the healthy gut microbiota within certain parameters because it is essential for the absorption of nutrients, improvement of digestion, vitamin synthesis (Jones and Bevins 1992; Lehrer and Lu 2012) and also the inhibition of pathogen growth (Sekirov et al. 2010). On the other hand it has to tightly control the commensals and must protect the mucosa from invading pathogens (Eberl 2010).

The crypts of Lieberkühn (figure 2), named after its discoverer Nathanael Lieberkühn (1711–1756), are home to enterocytes characterized by a luminal brush border absorbing electrolytes and water, mucus-secreting goblet cells, enteroendocrine cells, which secrete different hormones and tuft cells which sense substances of the lumen and Paneth cells. Paneth cells (PCs) are specific to the small intestine, where they are located at the bottom of the crypt in close contact to stem cells from which the develop (Clevers 2013). Their main role is to produce antimicrobial peptides (Jones and Bevins 1992; Lehrer and Lu 2012). This makes them important players of the innate immune defence in the small intestine. Their endogenously synthesized antibiotics can kill bacteria, viruses and fungi (Zasloff 2002). In many stages of the epithelial development, Wnt signaling plays an essential role, for example cell movement and polarity as well as tissue and cell type generation rely on this pathway (Verzi and Shivdasani 2008).

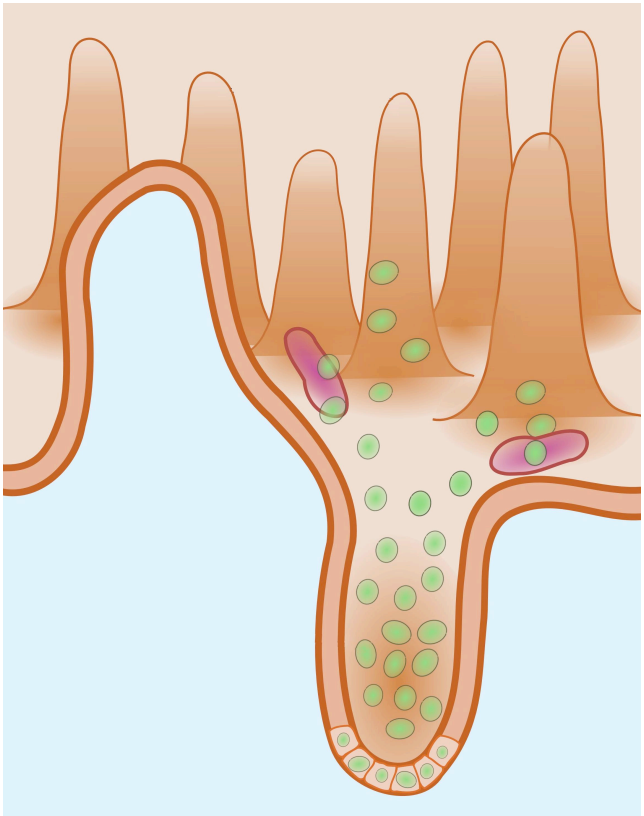

Figure 2: The Crypt: Paneth cells at the button of the crypt secrete defensins (green), mostly the α-defensins human defensin 5 and 6, to protect the host against pathogens and shape the microbiota (pink) in the small intestine (Teltschik et al, unpublished).

1.2.2 Interplay with gut microbiota

After a very variable time in early colonization in first months of life of the host, most individuals share a core composition of bacterial genera (Palmer et al. 2007). In later life, shaping the gut microbiota depends on many factors. There are three main parts: nutrition, immune development and innate host defence (O'Hara and Shanahan 2006). Therefore host health is highly dependent on a working interaction between gut bacteria, dietary nutrients and surface tissues in the gut. As an important environmental aspect, the human diet has influences on shaping the microbiota in the gut, which is known from different studies (Walter and Ley 2011; David et al. 2014). Diarrhoea and inflammations are common problems in people with malnutrition as well as bacterial translocation, which could also be linked to decreased levels of antimicrobial peptides (Hashimoto et al. 2012. 2; Hodin et al. 2011; Teltschik et al. 2012). In this context it is interesting to know that, under a lack but also abundance of nutrition, Paneth cells show decreased expression of antimicrobial peptides and aberrations in their granula (Hodin et al. 2011; Yilmaz et al. 2012). In turn, host metabolism relates to a certain part on the metabolism of gut microbes, enabling the host to use bacterial pathways for the absorption of dietary components (Nicholson, Holmes, and Wilson 2005). The microbiota takes the task of synthesizing vitamins and ferments non-digestible parts of the nutrition. K and B vitamins as well as essential amino acids and short-fatty acids must be supplemented to the feed of germ- free mice (Wostmann 1981; Gustafsson 1982; Hooper and Gordon 2001). The co-evolution of bacteria and men made it possible to use other sources to cover nutrient requirements, which were not available before. However, the bacterial site is also profiting from this relationship; it gained a niche full of nutrients, which facilitates bacterial growth as well as stable temperature and redox potential conditions. This in turn enables the beneficial microbiota to defend their habitat against invading pathogens (Hooper, Midtvedt, and Gordon 2002; Salyers and Pajeau 1989; Hultgren et al. 1993). The commensal microbiota is also involved in the regulation and development of the mucosal immune system (Cerf-Bensussan and Gaboriau-Routhiau 2010). During postnatal colonization all microbes have to compete for nutrients and have to resist the host's antimicrobial defenses. For establishing a stable microbial composition, it is essential to form cross- feeding networks, where metabolites from one bacterial group act as substrates for other groups (Duncan et al. 2004). There is more and more evidence that disturbances in this stable microbial composition can promote the development of diseases (Neish

2009). Disturbed microbial colonization, called dysbiosis, has been linked to different diseases like inflammatory bowel diseases, obesity and even colon cancer (Bäckhed et al. 2007, 2004; Azcárate-Peril, Sikes, and Bruno-Bárcena 2011). This shows the importance of a better understanding of the molecular mechanisms behind these interactions.

1.2.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are found in different protein families and the most prominent ones are the defensins and cathelicidin (LL-37), c-type lectins (e.g. the Reg family), ribonucleases and S100 proteins (e.g. calprotectin) in the intestinal tract (Harder, Gläser, and Schröder 2007; C L Bevins 2003). Reduced antimicrobial defence in the gut can promote chronic inflammation (Wehkamp et al. 2008). These effects can be seen in inflammatory bowel diseases (IBD) and especially in Crohn´s disease (Koslowski et al. 2010). This demonstrates that AMPs are crucial to maintain a health promoting balance and a beneficial homeostasis between the microbial gut community and the host. AMPs are produced and secreted on-going or also on demand (Salzman et al. 2010). Transgenic human defensin 5 (HD-5) mice, which exhibit an altered antimicrobial activity, are protected against *Salmonella* infections (Salzman et al. 2003) and a significantly changed expression of α-defensins, either diminished or increased, lead to significant changes in the composition of the intestinal microbiota. This data demonstrates their *in vivo* homeostatic role towards the symbionts (Salzman et al. 2010).

1.2.3.1 Defensins

The predominant antimicrobial peptides in the gut are the defensins. Their outstanding role for an effective defence against bacteria, fungi, and some enveloped viruses in the intestine makes them essential to maintain a proper composition of the microbiota and restricting contact between resident microbes and mucosal surfaces (Wehkamp, Schauber, and Stange 2007). They are the most extensively studied intestinal antimicrobial peptide group and therefore will be explained in detail in this chapter. Their mechanism of action, functions and expression will be elucidated.

Data from mouse models illustrated their importance in controlling the intestinal mucosal barrier and protecting the organism from pathogens. For example does an extensive release of Paneth cell antimicrobials, called degranulation, induced via TLR9 stimulation, protect mice against an infection with the bacteria *Salmonella typhimurium* (Rumio et al. 2004) as their transcriptional induction and secretion is

at least in part dependent of innate immune PRRs. In contrast, a lack of the metalloproteinase matrilysin, an enzyme which activates cryptdins (mouse Paneth cell α-defensins), leads to a high susceptibility to orally administered pathogens (Wilson et al. 1999).

Paneth cells, which are specialised cells, located at the bottom of the crypts of Lieberkühn in the small intestine (figure 2) mostly secrete α-defensins. With their broad antimicrobial spectrum they are one of the major producers of AMPs in the small intestine (Wehkamp et al. 2006), actively contributing to the mucosal immunity by secreting e.g. lysozyme, phospholipase A2 group IIA or REGIII, but their main products are human α-defensin 5 (HD-5) and 6 (HD-6) (Clevers and Bevins 2013a). HD-5 and HD-6 not only have antimicrobial but also antiviral activity (Doss et al. 2009; Klotman and Chang 2006) and even anti-parasitic properties have been described for HD-5 (Ericksen et al. 2005). However, it is also described that they increase infectivity of some viruses (Klotman et al. 2008). The expression of these two antimicrobials is controlled by different signaling pathways, amongst others signaling induced by the stimulation of the intracellular receptor NOD2 (CARD15) or the β–catenin dependent Wnt signaling pathway (Koslowski et al. 2010) - but mainly assumed to be consistent. Although they have analogous 5' region and are therefore similarly regulated (Mallow et al. 1996), the mode of action of HD-5 and HD-6 differs from each other. HD-5 has direct microbial and antiviral effects and diminishes the survival of microbes by killing them even in low concentrations (Ericksen et al. 2005; Porter et al. 1997; Zins et al. 2014). It´s mode of action and the exact details are not completely understood, but it is believed to disrupt the bacterial cell wall due to its amphipathic properties (Wei et al. 2009). HD-6 entraps bacteria in nanonets and thereby disables them and keeps them from entering the gut wall (Chu et al. 2012). It could however also been shown that HD-6 can additionally elicit direct antimicrobial effects when reducing conditions are present (B. O. Schroeder et al. 2014). Deficiencies in antimicrobial peptide expression are associated with chronic inflammatory disorders like IBD, which is triggered by intestinal gut microbes (Wehkamp et al. 2004; Wehkamp et al. 2005).

In contrast to α-defensins, the β-defensins are more ubiquitously expressed and can be found in various epithelia within the whole body. Like the α-defensins, they are small and have a cationic character and they also hold an important role in combating pathogens, particularly in the colon (Peyrin-Biroulet et al. 2010). Colonic Crohn's disease (CD) is associated with decreased on demand inducibility and

secretion levels of human β-defensin 2 (hBD-2) (Wehkamp et al. 2003). Different from inducible β-defensins, hBD-1 seems to be stable in disease and inflammation but also shows low mRNA copies in colonic CD (Peyrin-Biroulet et al. 2010). HBD-1 is one of the most prominent β-defensins but shows low bactericidal activity under aerobic conditions. B. Schröder from our group could show that this defensin, under reducing conditions, develops strong antimicrobial activity against the anaerobic, Gram-positive commensals species *Bifidobacterium* and *Lactobacillus* and even the fungus *Candida albicans* (Schroeder et al. 2011).

1.3 Paneth cells

Discovered already in 1872 by G. Schwalbe, these cells were named after J. Paneth, who has performed their detailed morphological analysis in 1888. Their function was discovered almost 100 years later. Today investigations focus on their role in innate immune defence and their function in regulation of intestinal stem cells. The pyramidal-shaped Paneth cells (PC) have a basally situated nuclei and a granula-rich cytoplasma where they produce and store their antimicrobial peptides (Bevins 2004; Wehkamp et al. 2006). Like all epithelial cell they originate from stem cells, which are located near the crypt bottom (Cheng and Leblond 1974). Different to the other epithelial cells, which are produced in the lower part of the crypt and migrate towards the villus tip, PC reside at the bottom of the crypt (Heath 1996; Marshman, Booth, and Potten 2002) (figure 2). Where they stay in close contact with intestinal stem cells. The intestinal epithelium has a rapid and continuous renewal rate, in comparison to that, the lifetime of PCs is long: their life span is around 60 days, which is the only exception to the high renewal rate (Barker 2014; Ireland et al. 2005). PCs can be identified by histochemical and immunochemical methods. They are stainable with eosin and phloxine-tartrazine. This staining shows the cationic charge of the granula content, which is typical for antimicrobial peptides, especially defensins (Jones and Bevins 1992). Because staining depends on different factors like tissue fixation, disease state and species its not easy to prove presence of PC relying only on histological methods (Porter et al. 2002). The produced antimicrobials are released from PCs granula after the stimulation of pattern recognition receptor (PRR) such as TLRs or NOD receptors by bacterial components like muramyldipeptide (MDP), which is the peptidoglycan motif common in all bacteria (Ayabe et al. 2000; Girardin et al. 2003). In chronic inflammation but also in other diseases, metaplastic PCs appear in other locations than the small intestine, for example the colon or the

oesophagus. Their specific role is not clear but it is possible, if you consider the relevant role of PC, that they could be meant to improve the antimicrobial defence in these diseased parts of the body (Bevins and Salzman 2011). Beyond that metaplastic PCs are also often associated with colorectal cancer (Polakis 2000; Roth et al. 2012). Causative could be the often over-active Wnt signaling pathway, which is essential for proliferation (and thereby a prevalent theme in the emergence of cancer) but also for the maturation of PCs (van Es et al. 2005).

But PCs are not only essential for maintaining intestinal homeostastis; they also secrete factors, which are important for shaping the stem cell microenvironment, the so-called "niche". These mediators are epidermal growth factor (EGF), the Notch ligand Delta like ligand (Dll) 4 and also Wnt signals (Sato et al. 2011). These factors are essential for cultivating intestinal stem cells *in vitro*. That PCs are supporting the maintenance of the stem cell niche was also shown by results revealing that stem cells improve growth in cell culture when co-cultured with PCs. Due to the important function in regulating proliferation the number of PCs in one niche is strictly controlled and does not exceed 10 PCs/niche. The regulation of PC number is due to Wnt antagonists like Dickkopf (Dkk) proteins and negative feedback loops (Clevers, Loh, and Nusse 2014; Clevers and Bevins 2013b).

1.4 The Wnt signaling pathway

Different molecular signaling pathways are involved in regulating and maintaining epithelial cell polarity in the intestinal tract. Notch, hippo, transforming growth factor- β (TGF- β)/bone morphogenetic protein (BMP), hedgehog and the Wnt pathway regulate cell fate and self-renewing processes in different tissues in the gut. The Wnt signaling pathway is highly conserved in all animals and regulates fundamental cellular interactions in multicellular organisms. β-catenin dependent Wnt signaling is indispensable for stem cell proliferation and maturation of Paneth cells. It maintains an undifferentiated state of the intestinal crypt progenitor cells and has a central role in regeneration and differentiation of the intestinal epithelium (Gregorieff et al. 2005; Korinek et al. 1998; Koslowski et al. 2009). Wnt factors, which are produced and secreted by epithelial cells at the bottom of the crypt (Crosnier, Stamataki, and Lewis 2006), generate a gradient of Wnt signals along the crypt–villus axis. Wnt signaling (figure 3) is activated by binding of ligands to the Wnt receptor complex on the cell surface, which includes either transmembrane co-receptor Low-density lipoprotein receptor-related protein (LRP) 5 or 6, binding and

internalizing ligands in the process of receptor-mediated endocytosis, and their co-receptor family Frizzled (Zhong et al. 2012). This leads to phosphorylation of the cytoplasmatic tail of LRP5 or LRP6, which enables the stabilisation of β-catenin in the cytoplasma. Subsequently, this main transducer of the canonical Wnt signaling translocates from the cytoplasma into the nucleus and forms complexes with transcription factors of the LEF/TCF family to activate target gene expression. The stability of β-catenin is regulated by phosphorylation, ubiquitination and subsequently degradation by a cytoplasmatic deconstruction complex consisting of the protein Axin, adenomatous polyposis coli (APC), the kinases glycogen synthase kinase-3 alpha/beta (GSK-3) and casein kinase-1 (CK1). In case of degradation, CK1 and GSK3β phosphorylate threonine and serine residues in the amino terminal region of β-catenin (Kitagawa et al. 1999; Behari 2010).

The transcription factor TCF-1 (also known as TCF-7) belongs together with LEF-1, TCF-3 (TCF-7L1) and TCF-4 (TCF-7L2) to a family of DNA binding proteins (van Beest et al. 2000). TCF-1, best known for its crucial role in thymus development (F. J. T. Staal, Luis, and Tiemessen 2008), requires the interaction with β– catenin, which provides a transactivation domain, to become an active transcription factor (Behrens et al. 1996; van de Wetering et al. 1997). It has been described that TCF-1 regulates proliferation of epithelial cells (Gregorieff et al. 2005). Through alternate promoter usage, different TCF-1 isoforms are generated. These isoforms can be sub-grouped into dominant negative isoforms, lacking the N-terminal β-catenin interaction domain and active isoforms, which are able to transactivate target genes (Van de Wetering et al. 1996).

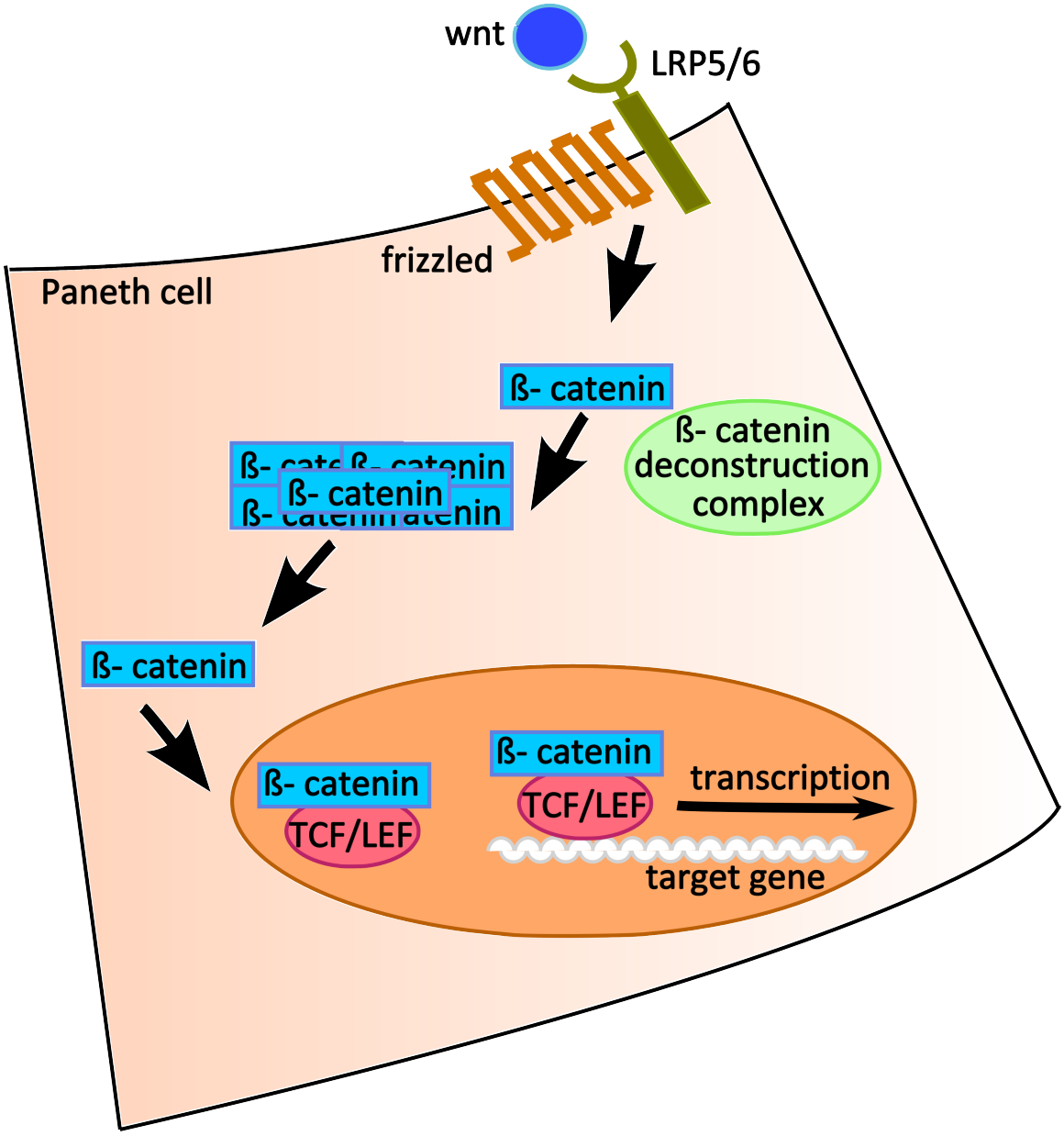

Figure 3: The Wnt signaling pathway in the Paneth cell
Interaction of Wnt proteins with receptors of the LRP and Frizzled family leads to pathway activation. This results in intracellular stabilisation of β-catenin due to decreased β- catenin degradation because of an inhibition of a β-catenin deconstruction complex. Stabilization enables β- catenin to enter the nucleus and interact with transcription factors of the Lef/TCF family. Target genes like human defensins 5 and 6 as well as other critical components of the complex Paneth cell gene program get activated (figure modified from Beisner, Teltschik et al., 2014, AJPGI).

Different model systems showed that disruption of β-catenin dependent Wnt signaling compromises Paneth cell differentiation (Korinek et al. 1998; Andreu et al. 2005, 2008). LRP5 knockout mice show normal development but have metabolic abnormalities and low bone mass (Kato et al. 2002; Magoori et al. 2003). In comparison to the LRP5 knockout model the depletion of the LRP6 gene is lethal (Pinson et al. 2000). Mice embryos with a double knockout in the LRP5 and 6 genes

die much earlier than LRP6 knockout mice, to be specific, during gastrulation (Kelly, Pinson, and Skarnes 2004). These studies show that LRP6 is more crucial in embryogenesis than LRP5, but they also show that LRP5 and 6 have some functional redundancy. Studies from our group revealed that a functional mutation, a non-synonymous single nucleotide polymorphism (SNP) (rs2302685; Ile1062Val), in the LRP6 gene is associated with early onset of Crohn´s disease. This SNP was former linked to increased fracture risk (van Meurs et al. 2006) and to an increased risk of myocardial infarction (Xu et al. 2014). This further strengthens the role of Wnt signaling in the pathogenesis of Crohn´s disease and further showed us the necessity to analyse the co- exressed receptor LRP5.

1.5 Crohn´s disease

Crohn´s disease (CD) is named after Dr. Burrill B. Crohn, who first described the disease in 1932 together with his colleagues Dr. Leon Ginzburg and Dr. Gordon D. Oppenheimer. The disease is an inflammatory disease of the gastrointestinal tract and belongs together with other diseases, namely ulcerative colitis (UC) and colitis indeterminata, to the group of inflammatory bowel diseases (IBDs). CD affects about 300 cases per 100000 people in Europe, with an annual incidence of 0.3-12.7 new cases per 100000 people (Preiß et al. 2009; Molodecky et al. 2012). The peak age-specific incidence occurs between 15 and 34, so patients often are affected the whole working life. The outcome are not only direct costs for drugs, consultations, hospital stays or even surgeries, but also indirect costs for non-productive time or the inability to work arise (Kappelman et al. 2008).

CD can affect any part of the gastrointestinal tract but most commonly it affects the ileum and the upper part of the colon. It can occur in a patchy pattern, which means that healthy parts are located between diseased areas. Often it results in strictures, microperforations and also fistulae. Histological manifestations are non-necrotizing granulomas, transmural lymphoid aggregates and fissuring or microscopic skip lesions (Podolsky 2002). A distorted epithelial structure, infiltration of plasma cells and lymphocytes, crypt abscesses, and lymphoid aggregates are characteristics to distinguish between IBD and other inflammations in the gastrointestinal tract (Le Berre et al. 1995). Typical symptoms are persistent diarrhea, rectal bleeding, pain and cramps in the abdomen and urgent needs for a bowel movement. Problems can also occur extra-intestinal and become manifested in symptoms like axial and peripheral arthritis, Erythema nodosum and growth

retardation in children. Medication and dietetic treatment can however weaken the symptoms and thereby improve quality of live. If cortisone, which attenuates the inflammation, is ineffective, immunosuppressant drugs like Azathioprine and 6-Mercaptopurine can dampen the immune response and thereby weaken the symptoms. Side effects of these drugs are often massive and patients are more prone to develop serious infections due to the weak immune defense (Lémann et al. 2006; Kübler et al. 2009). No curative medication is available so patients suffer from the disease lifelong.

The location of chronic inflammation is used to subcategorize disease subtypes. It can affect only the small intestine, which is categorized as L1, only the colon (L3) or both locations together (L2). Additional to an initial L1, L2 or L3 disease location, the upper GI tract can also be involved (L4). But not only the location is defining in CD, also the progression status can be used for classification; inflammatory or B1 is the weakest state, followed by B2 (stenotic) and a penetrating course (B3) with intestinal fistulas. Different from the location, the progression status can change over time. This makes it more likely that the mechanisms causing CD might be location-specific and therefore different in colonic and ileal CD (Silverberg et al. 2005; Louis et al. 2001; Jakobsen et al. 2014; Gasche and Grundtner 2005).

The pathogenesis of CD is not completely understood. Today, much data demonstrates that the immune system and also genetic predispositions as well as environmental factors like the gastrointestinal microbiota, stress, and diet are crucial in the development of IBD (Wehkamp et al. 2008; Hill and Artis 2010) and the normal gut microbiota plays an important causative role in the disease (Ostaff, Stange, and Wehkamp 2013).

1.5.1 Large intestinal Crohn's disease

Colonic CD is associated with reduced antimicrobial activity against certain strains of gut bacteria (Nuding et al. 2007). The mechanisms behind this reduction of antimicrobial defence are poorly understood, but it is believed that a lack of β-defensin induction could account for this disease pattern (Wehkamp et al. 2008). The production and secretion of human β-defensin 2 (hBD-2) needs to be induced by pathogens but also by probiotic bacteria. This induction is mediated via the "nuclear factor kappa-light-chain enhancer of activated B cells" (NF-κB) and activator protein 1 (AP-1) (Wehkamp et al. 2003). Especially the inducible hBD-2 is decreased in colonic CD as compared to ulcerative colitis. Impaired upregulation of hBD-3 and

hBD-4 could additionally contribute to impaired defence (Nuding et al. 2007; Fahlgren et al. 2003; Wehkamp et al. 2003). Other antimicrobials like Elafin, LL37 and SLP1 can be reduced in colonic CD and thereby account for disease (Schmid et al. 2007; Wehkamp et al. 2008). A lack of hBD-1, which is, in comparison to hBD-2, constitutively and widely expressed as one of the most prominent defensins (Bjoern O Schroeder et al. 2011), leads to decreased microbial activity in the colonic mucosa resulting in increased mucosal adherence of bacteria but also fungi or viruses (Swidsinski et al. 2002). This lack of hBD-1 can partly be explained by a functional SNP in the promoter of the hBD-1 gene, which seems to be predisposing for colonic CD (Kocsis et al. 2008; Peyrin-Biroulet et al. 2010). Other regulatory factors can also influence the expression of this important defence molecule. There are data available showing that the nuclear receptor PPARγ seems to be important for the maintenance of a constitutive epithelial expression of β-defensins in the colon, including hBD-1 in humans and mDefB10 in mice. Despite of its high expression and wide distribution, hBD-1 was long believed to be a very weak antimicrobial. This was due to the fact that defensins were studied under aerobic conditions. Schroeder et al. from our group could show that activation of hBD-1 depends on a reduction of its disulphide bonds. This happens under anaerobic conditions in the colon and hBD-1 thereby becomes a very potent bactericidal substance with killing capacity against fungi and bacteria (Schroeder et al. 2011).

1.5.2 Small intestinal Crohn's disease

In comparison to ulcerative colitis but also to colonic CD, development of ileal CD is linked to diminished expression of other, specific, antimicrobial peptides; more precisely both Paneth cell α-defensins HD-5 and HD-6 are reduced (Wehkamp et al. 2005). The secretion of these two antimicrobials seems to be linked to the Nucleotide- binding oligomerization domain containing molecule (NOD) 2 (also known as CARD15) receptor. As a peptidoglycan receptor it is important for recognising and reacting against invading pathogens by activation of NF-κB and thereby making it responsive to bacterial lipopolysaccharides (Hugot et al. 2001; Ogura et al. 2001). It could be shown that a mutation in this receptor, which is predominantly found in antimicrobial peptide- producing Paneth cells (Lala et al. 2003) is linked to decreased HD-5 and -6 levels (Wehkamp et al. 2004; Wehkamp et al. 2005; Armbruster, Stange, and Wehkamp 2017). Studying these connections in mouse models is challenging due to variability in experimental setup (Shanahan et al.

2014). Along with this, disturbances in the processing of defensins, to be specific, the processing of the α-defensin HD-5 precursor via trypsin and other proteases, potentially increase the risk to develop CD by leading to lowered levels of functional peptides and thereby to a reduced antimicrobial defence (Elphick, Liddell, and Mahida 2008). Disturbed differentiation of PCs and an affected amount and distribution of these cells, can compromise production and secretion of defensins. T cell transcription factor 4 (TCF-4, TCF7L2), which is an important factor in the Wnt signaling pathway, is in turn important for PC differentiation and is reduced in ileal CD patients. This negatively affects PC differentiation and leads to decreased levels of HD-5 and -6 (Wehkamp et al. 2007). Also mutations in the TCF-4 promoter region have been associated with ileal CD (Koslowski et al. 2009). In comparison to the decreased expression of HD-5 and -6, which is by the way independent of inflammation and specific for small intestinal CD (Bevins, Stange, and Wehkamp 2009), the expression of other PC products is unchanged (Kübler et al. 2009; Wehkamp et al. 2002). Other genetic variants in the autophagy-associated gene ATG16L1, which is important for PC biology are also specifically linked to ileal CD (Cadwell et al. 2008; Deuring et al. 2014). Wnt signaling, which is crucial for epithelial differentiation and production of AMPs in the PC, is disturbed in ileal Crohn´s disease (Koslowski et al. 2012; Wehkamp et al. 2007); a mutation in the LRP6 gene is associated with early onset Crohn´s disease (Koslowski et al. 2012). LRP6 and LRP5 are co-expressed receptors, often acting together and are both needed to respond to some Wnt signals (Goel et al. 2012). To elucidate if LRP5 is therefore another important factor in the development of CD, SNPs in this gene were analysed. For this analysis SNPs which were already described to be linked to other diseases like osteoporosis, increased fracture risk (Urano et al. 2009; Korvala et al. 2012; Xiong et al. 2007) and also familial exudative vitreoretinopathy were selected (Jiao et al. 2004). Furthermore coding SNPs were chosen, which could influence the structure or function of the gene and SNPs in putative regulatory regions. Tag SNPs, which should cover the major haplotypes were additionally analysed.

1.6 Aim of the work/ Hypothesis

Ileal Crohn´s disease (CD) is characterized by inflammations of the intestinal mucosa. The patients suffer from diarrhea, abdominal pain, fever, bleeding, tiredness, anal fissures and weight loss. The disease is not lethal but the life quality of the patients is strongly affected. CD is not curable because neither medical nor surgical methods lead to a cure. In light of this knowledge, it seems crucial to find therapies to improve the quality of live or even find a cure for the patients. Accountable for the persistent inflammations is a diminished production of the Paneth cell products HD-5 and -6, enabling the microbial challenge to overpower the weakened innate defense allowing the gut flora to enter the epithelium. Various data from our group showed that deficiencies of those two defensins are linked to impairments in the Wnt signaling pathway (Beisner et al. 2014b; Koslowski et al. 2009; Wehkamp et al. 2007). Although it is clear, that Crohn´s disease is a multifactorial disease, involving environmental (e.g. microbes, nutrition), genetic (SNPs), and likely also epigenetic (miRNAs, methylation, acetylation) factors, many details and potent other factors are unclear. Despite the findings made, it is known that the underlying genetics are more important in disease etiology of CD than in ulcerative colitis (Ellinghaus et al. 2015).

The Wnt signaling pathway regulates fundamental cellular interactions in multicellular organisms. β- catenin dependent Wnt signaling is indispensable for stem cell proliferation and maturation of Paneth cells. It maintains an undifferentiated state of the intestinal crypt progenitor cells and has a central role in regeneration and differentiation of the intestinal epithelium (Gregorieff et al. 2005; Korinek et al. 1998; Koslowski et al. 2009). The pathway has to be very tightly regulated and even small disturbances can dramatically change the mucosal barrier. To better understand the relations between Wnt signaling and diminished antimicrobial host barrier further factors of the Wnt signaling were analysed.

The already described mutations in the Wnt pathway alone cannot explain HD-5 and -6 deficiencies (Koslowski et al. 2009, 2012) so there is need to reveal further genetic factors involved. A SNP in the Wnt pathway receptor LRP6 was former linked to early onset of Crohns disease (Koslowski et al. 2012). To further elucidate the role of LRP6, IHC methods could clarify the location of this upstream component and mRNA data could reveal possible expression differences in patients and healthy controls.

As a co-expressed receptor of LRP6, LRP5 is also an interesting upstream component. Both receptors are co-expressed and have partly overlapping functions. Genetic analyses could reveal if, like for LRP6, a genetic association for small intestinal CD might exist. MRNA analyses of the subgroups might clarify possible expression changes. In order to consider possible LRP5 effects on HD-5 and -6 expression, overexpression experiments in cell culture could be conducted to show effects on target genes HD-5 and -6.

Transcription factors of the TCF family are important components of the Wnt target gene regulation and maintain proliferation of small intestinal stem cells and Paneth cell differentiation. The mentioned lower HD-5 and -6 levels are partly caused by TCF-4 (Wehkamp et al. 2007; Koslowski et al. 2012). This work could help to better understand TCF-1 in Paneth cell gene regulation and its role in CD. Immunohistochemical methods may confirm the location of TCF-1 in the small intestine. Furthermore mRNA expression experiments will be conducted and a TCF-1 knock out mouse model will be analysed.

Trying to better understand the mechanisms causing impairments in the antimicrobial defense, animal models can help to study and understand the role of defensins *in vivo*. For this reason two different liver cirrhosis rat models will be analysed to better understand the role of defensins and other antimicrobial peptides *in vivo*. From former studies it is known that patients with liver cirrhosis suffer from severe infections and sepsis caused by bacteria originating from the normal gut flora (Wiest and Garcia-Tsao 2005; Steffen, Berg, and Deitch 1988). This case is comparable with the situation in CD. In both diseases commensals play a central role in the disease pathogenesis. Expression analyzes of different Paneth cell and non-Paneth cell products could explain if the situation is similar to the one observed in ileal CD patients and shed light on the role of defensins and other AMPs *in vivo*.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Patient samples

All healthy controls and patients included in the studies gave their written and informed consent after the study purpose, sample procedure, and potential adjunctive risks were clarified. The ethics committees of the Medical University Vienna, Austria, the University Hospital Tübingen, Germany and the University of Leuven, Belgium approved all studies. Subgrouping of included patient samples was done according to provided phenotype data, which was based on clinical, radiological, endoscopic and histopathological diagnoses at the respective IBD centres. Following the Vienna classification the patients were sub-grouped into three subgroups: patients who suffer from ileal disease only (L1), patients who suffer from colonic disease (L2) and patients that have ileocolonic disease (L3).

2.1.1.1 Samples for mRNA expression analysis

Tissue samples used for mRNA analysis were either part of the Stuttgart cohort or the Norwegian cohort. The Stuttgart cohort has been continuously collected at the Robert-Bosch Hospital, Stuttgart since 2001. Included patients and healthy controls were endoscoped for diagnostic reasons. Additional biopsies for study purposes were collected from the ileum, coecum, sigma and the rectum and immediately shock frozen and stored in liquid nitrogen. The Norwegian cohort comprises 36 pediatric patients with CD and 29 non-IBD controls (< 18 years (median age in the CD group: 13.8 years, in the Non-IBD group: 12.2 years)) (all based in the catchment area). Sample collection that took place from May 2005 and December 2007 was part of a larger study in Inflammatory Bowel South-Eastern Norway (IBSEN-II) and investigated immunological, genetic and environmental factors, which can all contribute to the pathogenesis of IBD. Several biopsies were taken from different segments during endoscopy for histological evaluation. Histopathological analysis of an expert pathologist (Lars Gustav Lyckander) confirmed IBD diagnosis according to the Porto criteria. As Non-IBD controls served children with no evidence of IBD or mucosal inflammation (with final diagnoses of irritable bowel syndrome or recurrent abdominal pain). Terminal ileum and colon ascendens biopsies were obtained from 36 patients with CD and from 29 non-IBD controls. If possible, biopsies were taken from non-inflamed mucosa but also from inflamed mucosa of IBD patients.

Altogether 15 small intestinal biopsies and 4 colonic biopsies were macroscopically inflamed. A pathologist performed histological grading of the taken biopsies. The degree of inflammation was determined differentiating between normal mucosa (score 0), no active inflammation (score 1), mild active inflammation (score 2), moderate active inflammation (score 3), and severe active inflammation staining (score 4). Classifying the patients with CD according to disease behaviour 30 pediatric CD patients with a non-stricturing and non-penetrating disease type (B1) and 6 with stricturing behavior (B2) were available.

2.1.1.2 Samples used in genetic analysis

To analyse a potential association of genetic variants in the LRP5 gene, the distribution of 45 SNPs in patients with CD, UC and unrelated healthy controls were evaluated. The analyses comprised 3 Caucasian DNA cohorts (Vienna, Stuttgart and Leuven). DNA was isolated at the respective centres from whole blood via standard procedures and provided as either liquid or dried samples. Phenotyping data, which was collected in our institute, was used to classify and group the patients. To exclude effects due to differences between subgroups, subgrouping was performed according to criteria like age, gender, age of onset of disease, behaviour and involvement of small intestine (L1), colon (L2) or both (L3) and additional involvement of the upper gastrointestinal tract (L4).

The Vienna and Leuven cohorts have been previously studied by our group and are described in more detail in Koslowski et al., 2012 . The Vienna cohort consists of ~700 IBD patients (~200 ileal CD) which are compared to ~925 healthy blood donor controls from Stuttgart. The Leuven cohort consists of ~650 samples (300 controls and 350 IBD patients, including about 200 ileal CD cases). The DNA cohort from Stuttgart has been collected as part of a biobank assembly and measured ~500 samples. These included 148 control patients, who underwent routine endoscopy, 163 ileal CD (75 L1. 88 L3), 140 UC and 37 colonic CD patients.

2.1.2 Animal models

2.1.2.1 Tcf-1 knockout mice

Tcf-1 knockout and wild type littermate mice with a C57BL6 background were kindly provided by the group of Prof. Staal from the Utrecht University, Netherlands. The knockout was described previously (Verbeek et al. 1995). The mice were kept in the Utrecht University shared animal facility according to government regulations.

Tissue samples from the proximal, middle and distal part of the small intestine from Tcf-1 -/- (n=6), +/- mice (n=6) and control Tcf-1 +/+ mice (n=6) was available.

2.1.2.2 Rat models

Liver cirrhosis and portal vain ligation rat model were kindly provided by the group of Prof. Wiest from the University Hospital Regensburg, Germany.

All experimental procedures in this study were conducted according to the American Physiological Society principles for the care and use of laboratory animals and the local ethical committee approved the study. Tissue samples from the proximal, middle and distal part of the small intestine from rats with portal vain ligation (n=6) and healthy control mice (n=6) was analysed.

2.1.2.2.1 Prehepatic portal hypertension rat model

Induction of prehepatic portal hypertension (PVL): In order to examine whether the changes in antimicrobial peptide expression could be related to the phenomenon of portal hypertension per se the PVL model was chosen. This model is known to lack hepatic parenchymal cell damage as well as Kupffer cell dysfunction. Importantly, this PVL animal model is characterized by a high rate of bacterial translocation (BT) to mesenteric lymph nodes (MLN) at 2 days after PVL whereas in the chronic phase (two and more weeks after PVL) no BT is observed. Portal hypertension was induced surgically in aseptic conditions as it has been described before (Lopez-Talavera et al. 1996). Briefly, the rats (n=6) were anaesthetized with ketamine hydrochloride (Ketalar, 100 mg/kg body wt; Parke- Davis, Avon, CT, USA). After a midline abdominal incision, the portal vein was cleared from surrounding tissue. A ligature (silk gut 3-0) was placed around a 20-gauge blunt-tipped needle lying alongside the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. In sham-operated rats, the same operation was performed with the exception that after isolating the portal vein no ligature was placed. After the operation, the animals were housed in plastic cages and allowed free access to rat food and water. All studies were performed in 12-18 h fasted animals 2 days after surgery. Tissue samples from the proximal, middle and distal part of the small intestine from rats with portal vain ligation (n=6) and healthy control mice (n=6) were analysed.

2.1.2.2.2 CCl4-induced liver cirrhosis rat model

Cirrhosis was induced in male pathogen free CD rats (Charles River, 50-80 g initial weight) by inhalation of CCl_4 along with phenobarbital (0.35 g/l) in the drinking water, as previously described (Wiest et al. 2003). CCl_4 administration was started three times a week over one minute and increased every other week by one minute to a maximum of 5 minutes, depending on the animal's change in body weight. After 12 to 16 weeks this approach induces micronodular liver cirrhosis with ascites. Seven days prior to experimental procedures application of CCl_4 as well as phenobarbital was stopped. Only cirrhotic animals with decompensation of liver function and thus presence of ascites were used. Phenobarbital-treated age- and sex-matched rats were used as control group. Tissue samples from the proximal, middle and distal part of the small intestine from rats with portal vain ligation (n=6) and healthy control mice (n=6) were analysed.

2.1.2.2.3 Assessment of bacterial translocation

Experiments were performed under strict sterile conditions. Ketamine hydrochloride (Ketalar, 100 mg/kg body weight) was used as anesthesia. Rats were shaved and the skin was disinfected with alcohol. After midline laparotomy, MLNs draining lymph from the terminal ileum, coecum, and ascending colon were subsequently dissected, removed, and weighed (E400D scale from Ohaus Corp., Florham Park, NJ; accurate to ++ 0.01 g). Liver and spleen tissue were also removed and weighed. MLNs, liver and spleen samples were diluted in phosphate-buffered saline (0.1 ml per 0.1 g), homogenized and 100 µl of suspension was cultured on MacConkey, Mueller-Hinton and whole blood agar for 48 hours. Bacterial growth was considered as evidence of BT to MLNs. To exclude bacteremia, 3 ml of blood was withdrawn from the vena cava inferior and inoculated into aerobic and anaerobic Bactec culture bottles, which were incubated at 35 °C; the growth value (measuring the bacterial CO_2 production) was continuously monitored for at least 7 days. No bacterial growth was observed; confirming that this model of CCl_4- induced liver cirrhosis does not present spontaneous bacteremia.

2.1.2.2.4 Histological examation

Cross sections of the distal ileum, coecum and colon were fixed in 10% buffered formalin and stained with haematoxylin-eosin. To quantify the histological damage in intestinal tissue, a previously described scoring system was applied (Teltschik et al. 2012). Both the degree of inflammatory infiltrate and mucosal architecture were independently graded from 0 to 4 and the mean score was noted (figure 22). Histological analysis was performed in a blinded fashion.

2.1.3 Consumables

Table 1: Consumables

Antibodies	
Biotinylated anti-rabbit Ig G antibody (goat)	Vector, Burlingame, CA, USA
Horse-radish-peroxidase -labelled secondary antibody	DAKO, Glostrup, Denmark
LRP6 antibody	ABGENT, San Diego,CA, USA
TCF-1 antibody	Cell signaling, Danvers, MA, USA
Chemicals	
3'-Diaminobenzidine tetrahydrochloride	DAKO, Glostrup, Denmark
100bp and 1kb ladder	Invitrogen Corporation, Carlsbad, CA, USA
Agarose (NEEO ultra quality)	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Bromophenol blue	Merck KgaA, Darmstadt, Germany
Chloroform	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Cytoseal	Thomas Scientific, Swedesboro, NJ, USA
DMEM (Dulbecco's Modified Eagle Medium)	Gibco, Invitrogen, Karlsruhe, Germany
Diethylpyrocarbonate (DEPC)	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Dimethylformamide	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Dinatrium salt dihydrate (EDTA)	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
EcoRI time saving high fidelity restriction enzyme/ buffers	New England Biolabs (NEB), Ipswich, MA, USA
EDTA	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Ethanol	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Ethidium bromide	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ficoll	Sigma-Aldrich Chemie GmbH,

	Steinheim, Germany
Formaldehyde	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formamide	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
FuGENE 6 reagent	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
HCl	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Isopropyl alcohol	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Ketamine hydrochloride	Parke- Davis, Avon, CT, USA
Li Chrosolv water	Merck KgaA, Darmstadt, Germany
Luria Broth medium for cultivation in falcons	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Luria-Agar for plates	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Lysis buffer	Promega Corporation, Madison, WI, USA
MOPS (C7H15NO4S)	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
NEO-CLEAR®	Merck KgaA, Darmstadt, Germany
PBS buffer	Gibco, Invitrogen, Karlsruhe, Germany
Peroxidase blocking solution	DAKO, Glostrup, Denmark
Schaedler broth	Becton Dickinson, Sparks, MD, USA
Sodium actete	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Merck KgaA, Darmstadt, Germany
Sucrose	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Sterile nuclease free water	Delta Select, Dreieich, Germany
Stop&Glow	Promega Corporation, Madison, WI, USA
Tris	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Tris/EDTA buffer pH 9	DAKO, Glostrup, Denmark
TRIzol™	Invitrogen Corporation, Carlsbad, CA, USA
Tween 80	Merck KgaA, Darmstadt, Germany
Unspecific serum from a pig	Slaughterhouse, Stuttgart
X-Gal	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
Xylene cyanol	Merck KgaA, Darmstadt, Germany
Consumables	
50 ml Centrifuge Tube	Corning Incorporated, Corning, NY, USA
8-channel pipettes	Eppendorf AG, Hamburg, Germany
Anaero GenTM sachet	Oxoid Limited, Hampshire, UK

Anaerobe indicator	Oxoid Limited, Hampshire, UK
Columbia blood agar Plates	Becton Dickinson, Franklin Lakes, NJ, USA
Cryo tubes	Nunc/Thermo Electron LED GmbH, Langenselbold, Germany
Cover slip	R. Langenbrinck, Emmendingen, Germany
Eppendorf tubes (1.5. 2 ml, Safe look tubes)	Eppendorf AG, Hamburg, Germany
Genetic analyzer tubes and septa	Applied Biosystems, Carlsbad, CA, USA
Indicator paper	Macherey-Nagel GmbH & Co. KG, Düren, Germany
LightCycler® 480 Multiwell Plates 96	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Luminometer tubes	Sarstedt AG & Co., Nümbrecht, Germany
Multiflex Round Tips	Sorenson, Salt Lake City, UT, USA
Object plate	R. Langenbrinck, Emmendingen, Germany
Petri dish	Greiner Bio-One, Frickenhausen, Germany
Pipet tips: epT.I.P.S (different sizes)	Eppendorf AG, Hamburg, Germany
Pipet tips: Multiguard, Barrier Tips (different sizes)	Sorenson, Salt Lake City, UT, USA
Pestle	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
RNase AWAY®	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
RNase free reaction tubes	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
safe seal tips	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Scalpel	Feather Safety Razor Co, Osaka, Japan
SpectroCHIP® arrays	Sequenom Industrial Genomics, San Diego, CA , USA
Ultrafiltration NucleoFast® 96 PCR manifolds	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Kits and Enzymes	
Avidin-biotin-blocking Kit	Linaris, Dossenheim, Germany
Agilent RNA 600 nano Kit	Agilent, Santa Clara, CA, USA
AMV Reverse Transcription System Kit	Promega Corporation, Madison, WI, USA
Big Dye Terminator V.1.1 Cycle Sequencing Kit	Applied Biosystems, Van Allen Way, CA, USA
Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse	Dako, Glostrup, Denmark
DharmaFECT® Duo Transfection Reagent	ThermoScientific, Waltham, MA, USA
Dual Luciferase Reporter Assay System	Promega Corporation, Madison, WI, USA

EcoRI time saving high fidelity restriction enzyme and buffers	New England Biolabs, Ipswich, MA, USA
EnVision™+ System Kit	DakoCytomation, Glostrup, Denmark
HotStarTaq® PCR Kit	Qiagen GmbH, Hilden, Germany
iPLEX™ Gold assay for SNP Genotyping	Sequenom Industrial Genomics, San Diego, CA, USA
LightCycler® 480 SYBR Green I Master	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
QIAamp DNA Blood Mini Kit	Qiagen GmbH, Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen GmbH, Hilden, Germany
QIAquik PCR Purification Kit	Qiagen GmbH, Hilden, Germany
RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany
TOPO TA Cloning Kit	Invitrogen Corporation, Carlsbad, CA, USA
Laboratory equipment	
ABI Prism™ 310 Sequencer	Applied Biosystems, Carlsbad, CA, USA
Bio Photometer	Eppendorf AG, Hamburg, Germany
Centrifuge 5415D	Eppendorf AG, Hamburg, Germany
CL- GS6R Beckman Coulter Centrifuge	Beckman Coulter, Inc., Brea, CA, USA
Compact Analyzer	Sequenom Industrial Genomics, San Diego, CA, USA
Consort Electrophoresis Power Supply	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Electrophoresis chamber B1A	Peqlab, PEQLAB Biotechnologie GMBH, Erlangen, Germany
Gradient cycler	MJ Research now Bio-Rad Laboratories, Hercules, CA, USA
Leica DM 4000 B microscope	Leica Microsystems, Wetzlar, Germany
LightCycler® 480 Real-Time PCR-System	Roche Diagnostics Deutschland, Mannheim, Germany
Liquid nitrogen tank	Messer Industriegase GMBH, Bad Soden, Germany
Luminometer AutoLumat *Plus*	Berthold technologies, Bad Wildbad, Germany
MassARRAY® Nanodispenser	Sequenom Industrial Genomics, San Diego, CA, USA
MassARRAY® Compact Analyzer	Sequenom Industrial Genomics, San Diego, CA, USA
NanoDropTM 2000	NanoDrop, Wilmington, DE, USA
PTC-225 Peltier Thermal Cycler	MJ Research now Bio-Rad Laboratories, Hercules, CA, USA
UV table and camera for the documentation of electrophoresis gels	Cybertech, Berlin, Germany
Veriti 384well PCR Thermal cycler	Applied Biosystems, Carlsbad, CA, USA
Video Monitor WV- BM 900	Panasonic, Secaucus, New Jersey, USA
Software	
Cell Quest Software	Becton Dickinson, Franklin Lakes, NJ,

	USA
Geneious pro V.4.8.5	Biomatters Ltd, Auckland, New Zealand
GraphPad Prism Ver. 4.0	GraphPad Software, Inc., La Jolla, CA, USA
Haploview	Broad Institute, Cambridge, MA, USA
Leica Application Suite V4	Leica Microsystems, Wetzlar, Germany
Light Cycler Software V.3.5	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
Primer3 (freely available software)	Steve Rozen and Helen J. Skaletsky (2000) (http://primer3.wi.mit.edu/)
Sequenom software SpectroTYPER	Sequenom Industrial Genomics, San Diego, CA, USA
Test for deviation from Hardy-Weinberg equilibrium/ association (freely available software)	Helmholtz Institute Munich (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl)

2.1.4 Media and buffers

50x TAE buffer
Used for the preparation of electrophoresis gels/ while electrophoresis in chambers.
242 g Tris
57 ml glacial acetic acid
100 ml 0.5M EDTA (pH 8.0)
add 1 l nuclease free H_2O

6x sample buffer
Used for gel electrophoresis
40 g sucrose
0.25 g bromophenol blue or xylene cyanol
50 ml H_2O (aqua dest.)

Haemalm:
Needed for counterstaining of nuclei.
1 g $C_{16}H_{14}O_6$ (Haematoxylin)
1 l H_2O (aqua dest.)
0.2 g $NaJO_3$ (Natrium iodate)
50 g $KAl(SO_4)_2$ (Potassium alum)
50 g $C_2H_3Cl_3O_2$ (Chloral hydrate)
1 g $C_6H_8O_7$ (Citric acid)
Stirring for 14 days on a magnetic mixer

LB-medium
Used for bacteria breeding.
20 g lysogeny (Luria) broth
500 ml H_2O (aqua dest.)
0.5 ml ampicillin solution

LB-medium for agar plates
Used for plating bacteria and selection of bacteria colonies carrying the desired plasmids.
40 g luria agar
1 l H_2O (aqua dest.)
Autoclaving and subsequent cooling down to 60°C

1 ml ampicillin solution (100 mg ampicillin/1ml Millipore H_2O)
Before utilization for plating: treating of the hardened LB with 200 µl X-Gal solution (40 mg/ml dimethylformamide) for blue-white selection.

Phloxine staining solution:
Used for staining of Paneth cells.
0.5 g Phloxine B
0.5 g of $CaCl_2$ (Calcium chloride)
100 ml H_2O (aqua dest.)

Washing buffer:
Used for washing steps in immunohistochemical staining.
0.15 M NaCl (Sodium chloride) in 5mM Tris-buffer+ 0.1% Tween 80, pH 7.6
For 2 l:
NaCl M= 58.44 g/mol
Set: 0.15 M NaCl → 17.532 g
Tris M= 121.14 g/mol
Set: 0.15 M Tris → 121.14/1000x2x5 = 1.2114 g
0.1% Tween 80 → 2 ml
Fill up to 2 l, adjust pH with HCl (Hydrochloric acid)
Store at 4°C

Primer and plasmids:
Primers are listed in table 2. The plasmids used as standards for calculating mRNA copy numbers were constructed using the PCR2.1-TOPO vector further described under 2.2.2.3 and the PCR products generated with the listed primers (table 2).

Table 2: forward and reverse primer used for qPCR and sequencing
Primers were designed with freely available Primer3 software (http://primer3.wi.mit.edu/).

Gene	Sense 5´-3´	Antisense 5´-3´	Product size (bp)
HUMAN			
β-actin	GCC AAC CGC GAG AAG ATG A	CAT CAC GAT GCC AGT GGT A	120
LRP-5	ACA ACG GCA GGA CGT GTA AG	GTC CAG CGA GAT CCT CCG TA	82
LRP-6	TGC CAT TGC CAT AGA TTA C	CCA TTG AGC CTT GTC ACT TC	219
TCF-1 iso 1	ACG AGC TGC TGG CCT TC	GTG GGT AAT GCA TGA GCA GA	336
TCF-1	CTC ATA AGT TGG ACC AGA GGA AG	GGC GGA CTG AAT GCT GAA AGA	429
HD-5	GCC ATC CTT GCT GCC ATT C	AGA TTT CAC ACA CCC CGG AGA	241
HD-6	CCT CAC CAT CCT CAC TGC TGT TC	CCA TGA CAG TGC AGG TCC CAT A	266
MOUSE			
β-actin	GCT GAG AGG GAA ATC GTG CGT G	CCA GGG AGG AAG AGG ATG CGG	100
Defa24	CAA GAG GCT GCA AAG GAA GAG AAC	TGG TCT CCA TGT TCA GCG ACA GC	93
Defa21	CCA GGG GAA GAT GAC CAG GCT G	TGC AGC GAC GAT TTC TAC AAA GGC	112
CRS-1C	CAC CAC CCA AGC TCC AAA TAC ACA G	ATC GTG AGG ACC AAA AGC AAA TGG	92
Lysozyme	GCC AAG GTC TAC AAT CGT TGT GAG TTG	CAG TCA GCC AGC TTG ACA CCA CG	86
TCF-1	CCA GGA CTC ACC CTC GT	TTC TGT GCC TAG CAA CCA A	220
Reg3γ	TTC CTG TCC TCC ATG ATC AAA A	CAT CCA CCT CTG TTG GGT TCA	101

RAT			
rβ-actin	GCT GAG AGG GAA ATC GTG CGT G	CCA GGG AGG AAG AGG ATG CGG	100
cryptdin 5	GAC CAG GTT GTT TCT GTC TCC ATT G	TGA GGC TTC CGT ATC TCT TGT TGC	152
cryptdin 7	AGC AAC CAT CAG ATG AGG ACC AGG	ACC TTG AGC ACA GAA CGC AGT GG	158
rHIP/PAP 3	CCA AGA ACC CAA CAG AGG TGG ATG	GGT CCC ACA GTG ACT TCC AGA GAC AG	106
r-lysozyme	CAA GCC ATA CAA TGT GCG AAG AGA G	TGT TGG TTT GAG GGG AAA GCA AG	220
rBD1	TCT GGA CCC TGA CTT CAC CGA C	TCT TCA AAC CAC TGT CAA CTC CTG C	246
rBD2	TTT CTC CTG GTG CTG CTG TCG C	CCA CAA GTG CCA ATC TGT CGA AAA C	131
rCRAMP	TGC CTC TAA CCG TTT CCC AGA CC	TGC TCA GGT AAC TGC TGT GAT GCC	214
rNP3	TTT GGA GGG GAT AAA GGC	TCA GCA ACA GAG TCG GTA	147
SEQUENCING			
T7	TAA TAC GAC TCA CTA TAG GG		
M13	CAG GAA ACA GCT ATG ACC		
LRP5 tf plasmide 1	CTC GTT CCG GCA GAA GGT		
LRP5 tf plasmide 2	ATG GGC CTC ATG TAC TGG AC		
LRP5 tf plasmide 3	CGA GTG TGC CAA CTT GGA T		
LRP5 tf plasmide 4	CAC AGG ATC TCC CTC GAG AC		
LRP5 tf plasmide 5	CTG CGC CTC ACA CTA CAC C		
LRP5 tf plasmide 6	GAA CGT CAA AGC CAT CGA CT		
LRP5 tf plasmide 7	CTG GAG GAC GCC AAC ATC		
LRP5 tf plasmide 8	AGA CTG TCA GGA CCGC TCA G		

2.2 Methods

2.2.1 Staining methods

2.2.1.1 Immunohistochemistry

LRP6 immunohistochemical staining was performed using the two-step immunoperoxidase EnVision technique. Human small intestinal tissue was fixed in buffered formalin and embedded in paraffin. Embedded tissue was cut in 3 μm sections, dewaxed with NEO-CLEAR® for 30 min and rehydrated in a graded alcohol series (100%-96%-70% (each step for one minute)) and subsequently rinsed in water. Slides were heated for 30 minutes in a steam cooker for antigen retrieval (pH 9) using a Tris/EDTA buffer pH 9, washed in water and endogenous peroxidase was blocked with peroxidase blocking solution. Subsequently slides were incubated for 1 hour with the primary anti-LRP6 antibody diluted 1:100 in TBST (20 mM Tris-Base (pH 7.4),

0.14 M NaCl, 0.1% Tween 20). Slides were washed in washing buffer and water and the horse-radish-peroxidase -labelled secondary antibody visualized the LPR6 protein. The secondary antibody was detected with 3'-diaminobenzidine tetrahydrochloride. Slides were washed with washing buffer and water and counterstained with hematoxylin (10 sec). Afterwards they were exposed to ascending alcohol series (70%- 96%- 100% (each step for one minute)) and mounted with Cytoseal. Sections were evaluated by light microscopy.

2.2.1.2 Avidin-Biotin-Complex (ABC) method:

Immunohistochemistry was performed using the EnVision™+ System Kit. Human small intestinal tissue was fixed in buffered formalin and embedded in paraffin. Embedded tissue was cut in 3 µm sections, dewaxed with NEO-CLEAR® for 30 min and rehydrated in a graded alcohol series (100%-96%-70% (each step one minute)) and subsequently rinsed in water. Endogenous peroxidase was blocked with peroxidase blocking solution. Unspecific reactions were blocked with unspecific serum from a pig (purchased directly from the slaughterhouse, Stuttgart). After every blocking step slides were rinsed with water. Slides were subjected to antigen retrieval in a steam cooker using a Tris/EDTA buffer pH 9. Slides were rinsed in washing buffer and subsequently in water. Endogenous biotin was blocked with an avidin-biotin-blocking-kit and subsequently rinsed with washing buffer and water. As primary antibody, the monoclonal TCF-1 antibody was used in a dilution 1:100 and incubated over night. Slides were rinsed with washing buffer and water and the biotinylated secondary antibody was incubated for 30 min. Slides were rinsed in washing buffer and water and ABC complex was incubated for 30 min. Subsequently slides were washed and DAB staining was performed (10 min). Slides were rinsed in water for 5 min. Counterstaining was performed with hematoxylin (10 sec). Slides were exposed to ascending alcohol series (70%- 96%- 100%) and mounted with Cytoseal. Sections were evaluated by light microscopy.

2.2.1.3 Phloxine-tartrazine Staining

Phloxine-tartrazine staining solution should not be older than two weeks. Human small intestinal tissue was fixed in buffered formalin and embedded in paraffin. Embedded tissue was cut in 3 µm sections, dewaxed with NEO-CLEAR® for 30 min and rehydrated in a graded alcohol series (100%-96%-70% (each step for one minute)) and subsequently rinsed in water. Slides were incubated with Haemalm for

5 min to stain the nuclei, after a 5 min wash in water the slides were incubated with the phloxine staining solution for 30 min. Slides were shortly washed in water (phloxine is water soluble) and then washed in 2-ethoxyethanol. Stripping was performed for 10 to 15 min in tartrazine (2.5 g of tartrazine in 100 ml in NEO-CLEAR®). Slides were rinsed and mounted with Cytoseal and sections were evaluated by light microscopy. Paneth cells appear in red/pink.

2.2.2 Molecular biological methods

2.2.2.1 RNA isolation, quantitative and qualitative analysis of isolated RNA, cDNA synthesis and real-time PCR

Because RNA is very sensitive to enzymatic or spontaneous hydrolysis, all RNA handling steps were performed with RNase-free materials. It is recommended to clean the workplace with isopropyl alcohol and RNase-away. RNA isolation was performed according to the manufacturer's protocol with the principle of acidic phenol/ chloroform partitioning of RNA into an aqueous supernatant: Frozen biopsies were pestled in liquid nitrogen and lysed in 100µl TRIzol™. TRIzol™ contains guanidinium isothiocyanate as a powerful protein denaturant for the inactivation of RNases.

After incubation for 10 min at RT, 200 µl of chloroform was added to the tissue- TRIzol™ mix. After another incubation for 3 min at RT and centrifugation (4°C and 11.6 rpm) 3 phases could be defined: one colour-less aqueous top phase containing RNA, a middle, grey DNA interphase and a red, organic, phenol-chloroform phase at the bottom containing lipids and proteins. The RNA containing phase was transferred into a Dolphin Eppendorf tube. Using 99.7% isopropyl alcohol RNA was precipitated, washed with 75% ethanol, dried and subsequently dissolved in 25µl 0.1% DEPC aqua dest., and stored at –80°C. Concentration of RNA was determined photometric with NanoDrop 2000 spectrophotometer and a quality check was performed with Agilent RNA 600 Nano kit and the Agilent 2100 bioanalyzer according to the manufacturer's protocol.

RNA isolated from cell culture experiments was extracted with the RNeasy Mini Kit according to the manufacture's protocol. Briefly cells were washed and lysed with RLT buffer. Subsequently the homogenate was centrifuged in the QIAshredder columns. To provide ideal binding conditions, ethanol was added to the lysate. The lysate was then loaded onto an RNeasy silica membrane and centrifuged. A DNase digestion step with DNase1-RDD buffer mix was performed. After a washing step

with RPE buffer RNA was eluted with RNase-free H_2O and stored at -80°C.

Total RNA was transcribed into cDNA with oligo (dT) primers and AMV-reverse transcriptase (RT) according to the manufacturer's protocol. Briefly 1 µg of total RNA was diluted to achieve a volume of 9.75 µl and incubated for 10 min at 70°C to linearize possible double strands. After adding 10.75 µl of a mix containing reaction agents according to the manual, the reaction was cycled for 15 min at 42°C and then for 5 min 95°C in a thermocycler PTC-200. Nuclease-free water was added to achieve an end volume of 100 µl. cDNA was stored at -20°C and used for the analysis with quantitative real time polymerase chain reaction (qRT-PCR) in the LightCycler 480 using primers, listed in table 2 for the different gene products and normalised to the house-keeping gene β-actin.

The real-time PCR relies on the same principles, like a common PCR, but it enables quantification of the PCR product with the help of a fluorescent dye. The LightCycler480 system uses SybrGreen, which can intercalate with double-stranded DNA and therefore quantify the amount of new synthesised DNA via the increase of emitted fluorescence. So it is possible to continuously monitor cycle by cycle accumulation of the fluorescently labelled PCR product. cDNA corresponding to 10 ng of RNA served as a template in a 10 ml reaction containing 4 mM $MgCl_2$. 0.5 mM of each primer and LightCycler-FastStart DNA Master SYBR Green I mix. Samples were loaded into 96-well plates and incubated in the LightCycler 480 with different cycle programs, depending on the individual melting temperature of the used primer pairs (see below). Quantification of the single PCR products and analysis of the standard curves obtained for each primer set (1 ng to 10^{-7} ng, in 1:10 dilution steps of DNA) was performed using LightCycler Software.

Table 3: Run templates for LightCycler480

denaturation	amplification	melting	products
95 10 min	95-60-72 1s - 10s - ∞	95-65-95 1s - 10s - ∞	rBD1, rCrypt5, rCrypt7, rHIP/PAP3, lysozyme, rNP3, rBD1, rBD2, rβ-actin, rCRAMP, rRELM, rPSP, β-actin, mReg3y
96 5 min	96-62-72 10s - 5s - 15s	95-60-95 1s - 15s - ∞	mTCF-1, m-lysozyme, mβ-actin, mCRS-1c, LRP6
97 5 min	96-64-72 10s - 5s - 15s	95-62-95 1s - 15s - ∞	LRP5
96 5 min	96-60-72 10s - 5s - 15s	95-58-95 1s - 15s - ∞	TCF-1
96 5 min	96-58-72 10s - 5s - 15s	95-56-95 1s - 15s - ∞	panCrypt
97 5 min	96-64-72 10s - 5s - 15s	95-62-99 1s - 15s - ∞	mDef21, mDefa24
99 5 min	99-62-72 10s - 5s - 15s	99-60-99 1s - 15s - ∞	TCF-1 isoform 1
95 5 min	95-66-72 10s - 5s - 10s	95-65-95 1s - 15s - ∞	HD-5, HD-6

2.2.2.2 DNA analysis from whole blood

Whole blood DNA from healthy controls and patients with IBD was isolated according to the manufacturer's protocol using the DNA blood purification kit (Qiagen GmbH Germany, 40724 Hilden, Germany).

2.2.2.3 Plasmid construction and restriction enzyme digestion

To evaluate expression levels of mRNA isolated from patient samples, plasmids to create standard curves were constructed. For amplification the primers listed in table 2 and HotStarTag DNA Polymerase according to manufacturer's protocol were used. cDNA samples from healthy control patients served as template. Product size and quality was confirmed with an agarose gel (1% agarose in 1x TAE buffer, 2 drops ethidium bromide), purified with the QIAquick PCR Purification Kit and concentration and quality was checked with the Agilent RNA 600 nano kit and the Agilent 2100 bioanalyzer. Subsequently the PCR product was incorporated in a PCR2.1-TOPO vector and the construct was transformed in TOP 10 *E. coli* cells described in the manufactures protocol (TOPO TA Cloning kits). Transformation solution was plated on LB ampicillin agarose plates. To identify clones carrying the inserted plasmid, plates were coated with 40 µl X-Gal solution (40 mg X-Gal/ml N,N-dimethylformamide). After over night incubation (37°C) blue (due to vector religation

the β-Galactosidase could convert the X-Gal) and white colonies (due to DNA insertion in the vector enzymatic degradation of X-Gal was impossible) were visible. White colonies were picked and incubated in 5 ml LB medium (with ampicillin (1 µl/ml)) over night (37°C). Isolation of plasmid DNA was performed using the QIAprep spin Miniprep kit according to manufactures protocol: cells were lysed and DNA was denatured with basic buffer. After adding neutralization buffer the DNA was centrifuged to eliminate the remaining cellular parts. To achieve higher purity, the DNA bound in the column was washed twice and subsequently eluted with nuclease-free H_2O. Concentration was determined with the Spectrophotometer NanoDropTM 2000. To confirm the correct plasmid size, the plasmids were digested with the restriction enzyme EcoRI: 500 ng plasmid DNA and 2 µl NEB buffer was filled up to a volume of 17 µl with nuclease-free H_2O and incubated (1 h, 37°C). Subsequently xylen-cyanol buffer was added and an agarose gel (1% agarose in 1x TAE buffer, 2 drops ethidium bromide) was used to visualise the plasmid sizes. Plasmids showing the right size were sequenced.

2.2.2.4 Primer

Primer used for the construction of plasmid standards, sequencing and real-time PCRs were designed using freely available Primer3 online software and are purchased from Eurofins MWG Operon (Ebersberg, Germany). Primers used for genotying are designed using the LRP5 reference sequence NT 033903, listed in the SNPdb (NCBI) and the specialized MassARRAY® Assay Design Software. These primers were purchased from Metabion International AG. All primers are listed in table 2.

2.2.2.5 Sequencing of plasmid standards and the LRP5 cell culture overexpression plasmid

Constructed plasmids were sequenced with the ABI Prism™ 310 Sequencer and the BigDye Terminator v1.1 Cycle Sequencing Kit to confirm the correctness of the sequences. The ABI sequencer works with the chain termination method (Sanger et al., 1977). Sequencing PCR was performed according to the manufacturer's protocol. Primers T7 and M13 (sequences listed in table 2) which are binding 5´ of the LRP5 insert, in the insert and 3´ of the insert were used for sequencing of plasmid standards. For cell culture overexpression plasmids, the according primers were used (see table 2). DNA was precipitated, washed and solved in nuclease-free H_2O. After

electrophoresis the chromatograms were analysed with the Geneious software (Biomatters, Auckland, New Zealand) and compared to reference sequences available at the NCBI (US National Library of Medicine National Institutes of Health) database homepage.

Sequencing PCR

96°C	1 min	
96°C	10 sec	24 cycles
50°C	5 sec	
60°C	4 min	
12°C	10 min	

Table 4: pipetting scheme for sequencing reaction

Ready Reaction Premix 1.1	2 µl
Big Dye Sequencing Buffer	1 µl
Primer forward or reverse (3.2pmol)	1 µl
DNA template	x µl (50 ng)
Nuclease-free H_2O	fill up to 10 µl

2.2.2.6 Genetic analysis

2.2.2.6.1 SNP selection in the candidate gene LRP5

Our group could formerly show that a rare variant of a coding single nucleotide polymorphism (SNP, figure 4) in the LRP6 gene is associated with early onset of ileal CD (Koslowski et al. 2012). In the present study focus was on its highly homogous co-expressed receptor LRP5 as a potential new factor in the development of ileal CD. Criteria for including SNPs were already described associations with other diseases (e.g. osteoporosis, increased fracture risk and familial exudative vitreoretinopathy), which were found in the NCBI database (SNPdb), coding SNPs which could influence protein stability and function and to further analyse the gene SNPs in the 3´- and 5´-region of the LRP5 gene were included. Additionally it was tried to cover the major haplotypes with Tag SNPs, which should predict the values of the remaining SNPs. A haplotype describes a collectively inherited and transmitted group of alleles. They can be used to discriminate between cases and controls in association studies and are necessary for linkage analysis. Location of Tag SNPs is primarily based on the principle of linkage disequilibrium (LD). If two SNPs are in LD there is a strong correlation between the alleles of these SNPs. LD describes the fact that two alleles in one population located at two or more gene loci occur non-randomly more often or less frequently as expected due to their individual allele frequency (Abdallah et al.

2003). Two statistical measures are used to describe the level of LD: D´ (deviation) is the difference between the observed and the expected frequency of one haplotype. LD intensity can be tested with D´: D´= 0 if the loci are in linkage equilibrium. If the loci are in linkage disequilibrium, then D´≠ 0. The correlation between two loci is described with r^2. r^2 = 0 indicates that two loci are in complete linkage equilibrium and r^2= 1 that the two loci are in complete linkage disequilibrium (Slatkin 2008). For selecting Tag SNPs the Tag-SNP picker function of the International HapMap Project homepage was used. This "Tagger" software, which is online available was developed by Paul de Bakker at the Center for Human Genetic Research of Massachusetts General Hospital, the Harvard Medical School and the Broad Institute (USA). Tag SNP selection was based on the CEU genotyping data which is freely available online on the HapMap homepage (www.hapmap.org), since all included study samples where patients and controls with a Caucasian background. Criteria additionally used for the SNP selection were a minor allele frequency cut off of 0.15 and an r^2 cut off of 0.8.

To ensure similar GC contents, the primer sequences were adjusted with preceding 5' ACG TTG GAT G 3' sequences. The manufacturer purified the primers by HPLC, desalted and scaled them at 0.04 µmol.

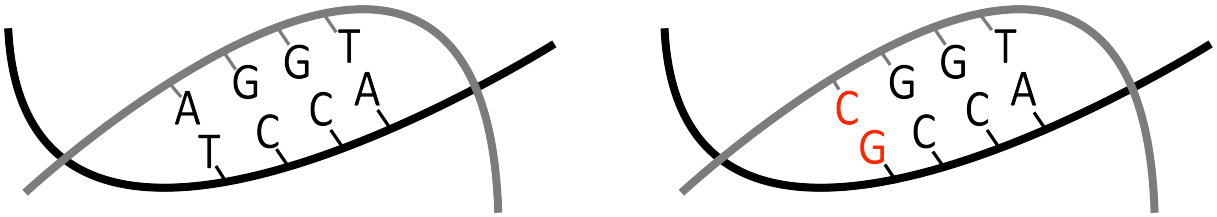

Figure 4: Single nucleotide polymorphism (SNP):
Schematic illustration of 2 DNA strains with differing base pairs: Left side: the major allele has A/T and at the same position the other strand (right side) is carrying the SNP and has C/G. SNPs can be synonymous (non coding, silent) and therefore lie outside a coding exon or non-synonymous and therefore resulting in a different amino acid after translation and lie inside a coding gene region (exon). If a SNP, which is lying in a coding region leads to a deletion or insertion of a single nucleotide it can cause a frame shift and thereby alter the transcription of the affected gene (Teltschik et al., unpublished).

2 µl DNA was pipetted in 384 well plates and dried overnight. Mastermix was mixed containing all needed primers and pipetted in a 96-well plate. 5 µl of the mastermix was added to the dried DNA and materials from the Sequenom iPLEX® Gold Kit were used according to the manufacturer's protocol. ~ 100bp fragments including the particular SNP were assembled with a Multiplex-PCR using the

HotStarTaq™ Master Mix according to the manufacturer's protocol and verified via gel electrophoresis. To verify the quality of the PCR products control samples were verified on a 3% agarose gel. The next step was a shrimp alkaline phosphatase (SAP) cleaning step to inactivate not incorporated remaining dNTPs. Patient samples were treated with the reaction mix and subsequently exposed to SAP reaction (table 6). Primer elongation (PEX reaction) was performed (for PCR conditions see below). The assay was designed in a way that the same conditions could be used for all primers, which are specifically designed and incorporated in a single termination mix (MassEXTEND®). This allows the detection of differences in mass at a single nucleotide level.

Patients from Leuven, Vienna and Stuttgart via MALDI TOF MS (matrix assisted laser desorption/ionisation time of flight mass spectrometry) were genotyped. This method enables analysis of single base polymorphisms (SNPs) in the genome (Griffin, Tang, and Smith 1997; Ross et al. 1998). A first primer elongation step was performed. For that step an extension primer was used which bound directly in front of the examined SNP in the PCR product. The reaction itself was carried out with three dideoxynucleotides which cannot be elongated after insertion and one deoxynucleotide (Sanger, Nicklen, and Coulson 1977). Because of the use of this reaction mix every extension primer can be elongated for one or two bases. Allele-specific DNA fragments with different lengths were generated.

To eliminate cationic molecules, which could disturb the MALDI TOF MS reaction a PEX reaction clean- up step with 6 mg Resin (Sequenom Industrial Genomics, San Diego, CA, USA) was performed. Nucleic acids show high affinity to alkaline and alkaline earth metal ions. Without this clean-up step additional signals would occur and disturb the MALDI TOF MS reaction. After adding 15 µl nuclease-free H_2O the plates were closed and shaken for 20 min subsequently they were centrifuged for 20 min (1300 rpm) and then spotted on SpectroCHIP® arrays utilizing the MassARRAY® Nanodispenser and analysed in the MALDI TOF MS mass spectrometer which can detect the masses and therefore the correlating genotype in real time. Analysis of the results, which were automatically loaded in a special database, was performed with the Sequenom software, which can automatically translate the masses of the single products into genotypes.

Table 5: Multiplex PCR reagents

(DNA was previously dried in 384 well plates and primers for each assay were previously mixed)

Reagent	Amount per well
HotStarTaq Master Mix	4 µl
Primer for (100.0 µM)	0.005 µl each
Primer rev (100.0 µM)	0.005 µl each
$MgCl_2$ (25mM)	0.3 µl
DNA (dried)	~ 10ng
Nuclease-free H_2O	Add 5 µl

Table 6: Reagents for SAP clean- up step

Reagent	Amount per well
Nuclease- free H_2O	1.53 µl
SAP	0.3 µl
10 x buffer	0.17 µl

Table 7: Multiplex PEX reagents mix (primers for each assay were previously mixed)

Reagent	Amount per well
iplex buffer	0.2 µl
iplex termination mix 0.2 µl	0.2 µl
Pex Primer (500.0 µM)	0.0112 µl each
iplex enzyme	0.041 µl
Nuclease- free H_2O	Add 2 µl

Thermocycler conditions for each reaction for preparation of PCR constructs for MALDI-TOF MS (MassARRAY® Compact System (Sequenom, San Diego, CA, USA))

Multiplex- PCR

Initiation	95°C	15 min	
Denaturation	95°C	30 sec	} 45 cycles
Annealing	56°C	60 sec	
Elongation	72°C	60 sec	
Final elongation	72°C	10 min	
Cooling	12°C	∞	

SAP clean- up step

1. Incubation	37°C	20 min
2. Incubation	85°C	10 min
Cooling	12°C	∞

PEX reaction

Initiation	95°C	4 min	
Denaturation	99°C	30 sec	56 cycles
Annealing	52°C	30 sec	
Elongation	72°C	30 sec	
Final elongation	72°C	2 min	
Cooling	12°C	∞	

2.2.3 Cell culture

2.2.3.1 Co-transfection

To analyse LRP5 and 6 effects on their potential target genes HD-5 and HD-6 cell culture experiments in HEK-293 cells were performed. A dominant negative and an active form of LRP6, which was co-transfected with either 1kb of the HD-5 or the HD-6 promoter was used. Béatrice Romagnolo and Pauline Andreu kindly provided the HD-5 and HD-6 luciferase reporter constructs which were described previously (Andreu et al. 2005). LRP5 and LRP6 expression plasmids were generously provided by Xi He and Mikhail V. Semenov (described previously (Tamai et al. 2004; Zeng et al. 2005)). The Wnt responsive TopFlash luciferase reporter construct was originally designed at the Hans Clevers lab. It contains multiple repeated copies of wild-type TCF/LEF-binding sites and can be induced via β-catenin-mediated transcriptional activation (Korinek et al. 1997; van de Wetering et al. 1997). It was gratefully received from Vladimir Korinek.

85000 HEK-293 cells were seeded in 24-well plates and grown for 24 hours. Subsequently cells were transfected with 200 ng of either the full-length LRP5 expressing vector, the full length LRP6 expression vector or a non-functioning dominant negative (dn) LRP6 expressing vector variant, or an empty vector as control, together with 200 ng of a TopFlash luciferase or HD-5 or HD-6 promoter construct and 50 ng of a Renilla luciferase expressing vector in each well, using the FuGENE 6 reagent according to the manufacturer's protocol. Cells were incubated for at 37°C for 48 h.

2.2.3.2. siRNA and co-transfection experiments

To reveal possible effects of the overexpression of LRP5, which could be covered by the stronger signaling activity of LRP6, LRP6 siRNA to knockdown the activity of intracellular LRP6 was used and LRP5 was overexpressed.

DharmaFECT® Duo Transfection Reagent which is designed for siRNA and plasmid co-transfection was used. 80000 HEK-293 cells were seeded in 24-well plates and grown for 24 hours. Subsequently cells were transfected with 75 ng of either the full-length LRP5 expressing vector or an empty vector as control, together with 375 ng of a TopFlash luciferase or HD-5 promoter construct and 50 ng of a Renilla luciferase expressing vector and 25 µl LRP6 siRNA (2 µM), a negative control siRNA (2 µM) or serum-free medium per well, using the DharmaFECT® reagent according to the manufacturer's protocol. Cells were incubated at 37°C for 48 h. Knockdown of LRP6 was confirmed by analysing mRNA expression levels of LRP6 with quantitative real-time PCR. Levels were at about 50 % lower than the expression levels in cells transfected with negative control siRNA or in control cells. One day post-transfection, cells were additionally stimulated with Wnt1 or Wnt3a to increase the induction activity of LRP5 (2.2.3.3).

2.2.3.3 Stimulation with either Wnt3a or Wnt1

Wnt3a and Wnt1, which are two ligands of LRP5, were used to stimulate and therefore increase the activity (signal transduction) of LRP5, this was previously shown by (Bhat et al. 2007). 24 h post transfection, the cells were stimulated with either Wnt3a or Wnt1: medium was removed and 500 µl of DMEM medium and 4.2 µl Wnt3a (100 ng/µl) or 1 µl Wnt1 (100 ng/µl) or 4.2 µl control medium (PBS+BSA) was added. Cells were incubated at 37°C for 24 h.

2.2.3.4 Dual Luciferase Reporter Assay

48 h post transfection, medium was removed from the wells, cells were washed with PBS buffer, and 100 ml passive cell lysis buffer diluted 1:5 in H_2O aqua dest. was added. Plates were shaken for 15 min and subsequently cells were resuspended by pipetting the cells up and down. 40 µl of the lysate was added to the luminometer tubes. LarII and Stop&Glow was added to the cell lysates according to the manufacturer's protocol and the luciferase activity was measured via the Dual Luciferase Reporter Assay with the luminometer AutoLumat *Plus* according to the manufacture's protocol. Firefly luciferase activity was assessed and normalized to the

corresponding studied promoter constructs. Increased activity of β-catenin dependent Wnt signaling in response to the overexpression of LRP5 or LRP6 constructs transactivates HD-5 or HD-6 promoter activity in the used HEK-293 cells. Values shown represent the average of triple determinations with the standard error of mean (SEM) indicated by error bars. Using the respective activity of the co-transfected *Renilla* luciferase, it was possible to normalize based on the transfection efficiencies. Three or more independent experiments were performed and transfections were carried out in triplicates.

2.2.4 Determination of antimicrobial activity with fluorescence activated cell sorting

Antimicrobial activity of defensins and other antimicrobial peptides can be assessed by fluorescence activated cell sorting (FACS). Antimicrobial activity from rat tissue was analysed as described before with modifications (Jan Wehkamp et al. 2007. 4; Nuding et al. 2006; Teltschik et al. 2012). Frozen tissue samples were pulverized with a pestle in liquid nitrogen and proteins were extracted under gentle agitation for 90 min in 60% acetonitrile + 1% trifluoroacetic acid. The acid soluble proteins in the supernatant were dried under vacuum and resuspended in 0.01% acetic acid. Mid-logarithmic growth phase suspensions of *Escherichia coli* K12 and *Enterococcus faecalis* ATCC 29212 were grown aerobically at 37 °C, whereas *Bacteroides fragilis* ATCC 25285 and *Bifidobacterium adolescentis* Ni3, 29c were cultured anaerobically (Anaero Gen; Oxoid Limited, Hampshire, UK). *Escherichia coli* K12, *Enterococcus faecalis* ATCC 29212, *Bacteroides fragilis* ATCC 25285 purchased from ATCC Bacteriology Collection and *Bifidobacterium adolescentis* Ni3, 29c isolated from stool of a child with disturbed small intestinal microbiota.

To verify that the acetic acid per se has no bactericidal effect, 10 µl bacteria suspension and the according volume of 0.01% acetic acid as negative/living control was used. Additionally pH-controls (pH 5, pH 4, pH 3, pH 2, pH 1 (60 % acetonitrile + 1% TFA in 0.01% acetic acid in increasing concentration) were used to exclude bactericidal effects due to acid- or acetonitrile remnants. As a positive control bacteria incubated with human β-defensin 3 (15 µg/ml bacteria suspension) were used.

Aerobic bacteria *(Escherichia coli* K12 and *Enterococcus faecalis* ATCC 29212) were incubated over night (37°C) on blood agar plates (Becton Dickinson, Sparks, MD, USA). The following day the optical density of the bacteria suspension was

tailored to OD_{600} = 0.05 with Schaedler broth 1:6 diluted in sterile H_2O (Becton Dickinson, Sparks, MD, USA). Subsequently bacteria were incubated for 1.5 h at 37°C until their growth reached OD_{600} = 0.1 (logarithmic growth). Anaerobic bacteria (*Bifidobacterium adolescentis* Ni3, 29c and *Bacteroides fragilis* ATCC 25285) were incubated in an anaerobic jar (Anaero Gen; Oxoid Limited, Hampshire, UK) over night (37°C) on blood agar plates. The following day they were diluted in Schaedler broth (1:6 diluted in sterile H_2O) and incubated for 48 h in the anaerobic jar at 37°C. This culture was then directly used (OD_{600}= 0.1).

Bacteria suspensions were diluted 1:10 with Schaedler broth (1:6 diluted with sterile H_2O) and incubated for 90 min at 37°C with the according protein mix. 1.5 µl $DiBAC_4(3)$ (1:50 in sterile H_2O) was added incubated for 5 min and centrifuged (5 min, 7000 rpm). The supernatants were discarded and the pellets were resuspended in 100 µl FACS- Flow and then added to 200 µl of FACS-Flow in FACS tubes.

Measurements were performed with the FACSCalibur™ cytometer (Becton Dickinson, Sparks, MD, USA). 30000-100000 events were analysed for every sample with the "Cell Quest" software.

2.3 Statistics

Data were analysed with GraphPad Prism 5. The values were tested for normal distribution (D'Agostino-Pearson test). Statistical analysis of real-time qPCR and antimicrobial assays were performed nonparametrically or parametrically (in case of normal distribution) by using the Wilcoxon U test, Mann-Whitney, or t test. Differences were considered significant at $p < 0.05$; values represent the mean of normalized data ± SEM. mRNA levels were normalized to β-actin and interpreted with the GraphPad Prism software.

The freely available Finetti based software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) which uses log the likelihood ratio chi square test, was used to test whether the SNPs are in the Hardy- Weinberg equilibrium. The same software was used to test for associations in subgroups (like age of onset, gender, location of disease) and controls in one cohort. Here the confidence intervals (CI) and odds ratios (OR) were calculated and Pearson's goodness-of-fit chi square tests were performed. Haplotypes and linkage disequilibria were calculated with Haploview.

3. RESULTS

3.1 Low-density lipoprotein receptor-related protein 6 (LRP6)

Parts of these results have previously been published in:

Association of a functional variant in the Wnt co-receptor LRP6 with early onset ileal Crohn's disease.
Koslowski MJ, Teltschik Z, Beisner J, Schaeffeler E, Wang G, Kübler I, Gersemann M, Cooney R, Jewell D, Reinisch W, Vermeire S, Rutgeerts P, Schwab M, Stange EF, Wehkamp J. PLoS Genet. 2012;8(2):e1002523. doi: 10.1371/journal.pgen.1002523. Epub 2012 Feb 23.

Author contributions:
Conceived and designed the experiments: MJK JW. Performed the experiments: MJK ZT MG GW IK JB. Analyzed the data: MJK ZT JB JW. Contributed reagents/materials/analysis tools: ES MG IK RC DJ WR SV PR MS EFS. Wrote the paper: MJK EFS JW. Recruiting and phenotyping of patients and controls in the association study: RC DJ WR SV PR. Obtained plasmids and performed pretests: GW. Involved in discussion of methods and data: JB ES MS.

As described previously the antimicrobial peptides HD-5 and HD-6 are reduced in ileal CD. Paneth cells in the small intestinal crypts exclusively produce these two defensins. Recently the β-catenin dependent Wnt transcription factor TCF-4 (TCF7L2) has been linked to an impairment in antimicrobial PC defense (Jan Wehkamp et al. 2007). To elucidate whether other upstream factors of the Wnt pathway could be involved in the regulation of PC defensins, it was now focused on LRP6. This co-receptor is expressed in the small intestinal epithelium (Zhong et al. 2012) and stabilises β-catenin which, after entering the nucleus (G. Liu et al. 2003; Semënov et al. 2001) binds to factors of the (Lef)/TCF family to activate target gene promoters including the HD-5 and -6 promoters (Giese, Amsterdam, and Grosschedl 1991; van Beest et al. 2000).

3.1.1 IHC staining shows LRP6 expression at the bottom of the crypts

Immunohistochemical (IHC) staining was performed using an antibody, which is directed against the C-terminal part of LRP6 or more precisely against the amino acids 1538- 1568 (figure 5). IHC staining revealed expression of LRP6 in the whole human small intestinal epithelium and therefore also in Paneth cells. This expression is consistent with former results from Zhong et al. and shows that LRP6 is expressed at the bottom of the crypt where Wnt signaling is important to regulate expression of its target genes, for example α- defensins.

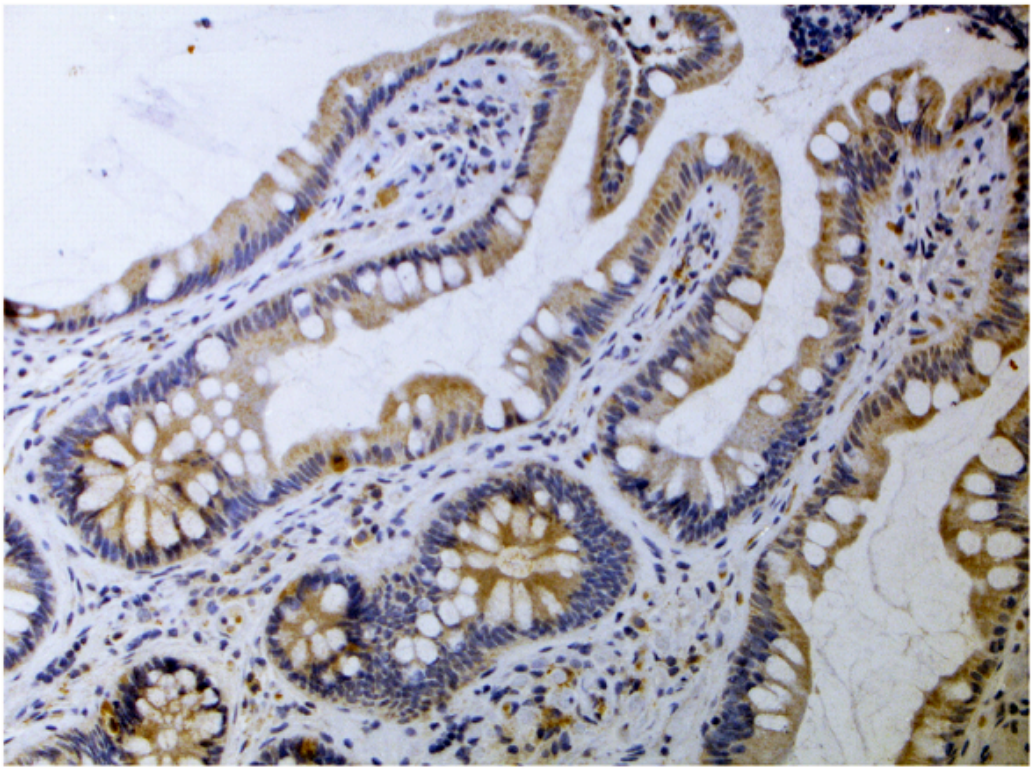

Figure 5: Immunohistochemical staining of LRP6 in the epithelium of human small intestinal tissue (Teltschik et al., unpublished)

3.1.2 Cell culture

3.1.2.1 Transcriptional regulation of HD -5 and -6 seems dependent on LRP6

Overexpression of LRP6 with a plasmid expressing the active form of LRP6 transactivates the human α-defensins HD-5 and -6 promoter in human embryonic kidney cells (HEK-293 cells). As seen in IHC staining experiments, the LRP6 protein is widely expressed in the human small intestinal epithelium (figure 5). Overexpression of LRP6 and co-transfection with TopFlash, that can be induced via β-catenin-mediated transcriptional activation, indicates an activation of β-catenin

dependent Wnt signaling as described before (Zeng et al. 2008; He et al. 2004). Overexpression of LRP6 and co-transfection with either HD-5 or -6 reporter plasmid leads to an induction of luciferase reporter activity (figure 6). In comparison, no effect was observed when using a non-functional variant of the LRP6 plasmid, which lacks the cytoplasmic part that is necessary for signal transduction or using an empty vector without the LRP6 insert as control. This shows that LRP6 is involved in HD-5 and -6 expression. Experiments were performed at least three times and transfections were carried out in triplicates. The values used are average with SEM indicated by error bars.

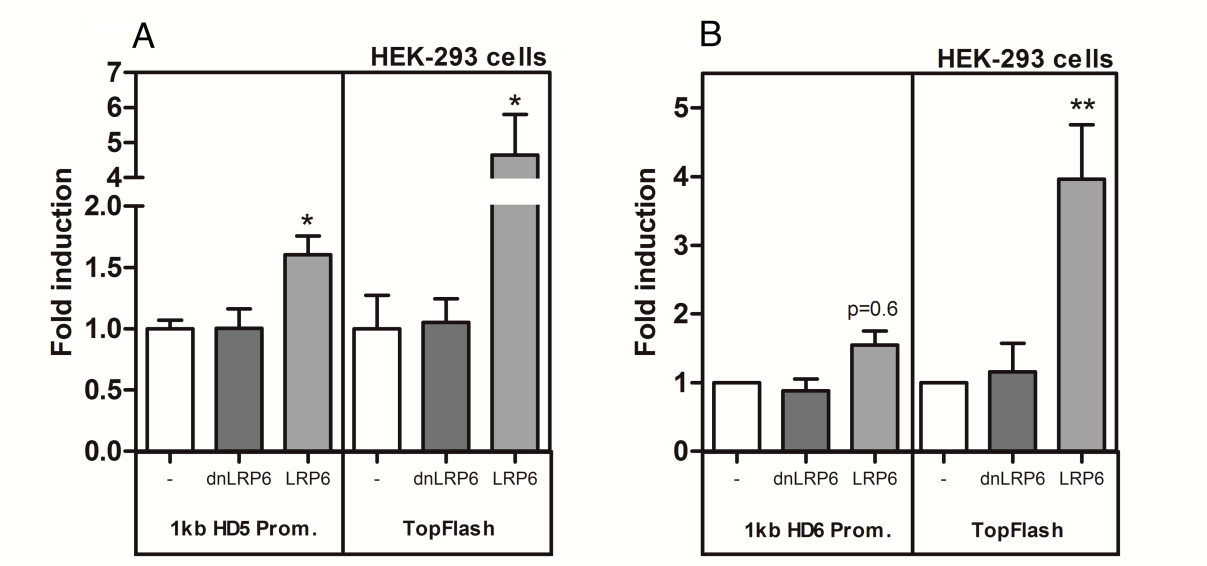

Figure 6: Increased activity of β-catenin dependent Wnt signaling via overexpression of LRP6 transactivates human α-defensin 5 (HD-5, graph A) and α-defensin 6 (HD-6, graph B) promoter activity in HEK-293 cells: Overexpression of LRP6 in HEK-293 cells leads to an activation of β-catenin dependent Wnt signaling as monitored by TopFlash activity (graph A, right panel and graph B, right panel). The transcriptional activity of a luciferase reporter under the control of either a 1kb HD-5 promoter or a 1kb HD-6 promoter was also increased after LRP6 mediated activation of Wnt. Both effects were not seen when a non-functional version of the LRP6 co-receptor (dnLRP6) was used. Values are the average of triple determinations with the SEM indicated by error bars, * $p < 0.05$, ** $p < 0.01$ (Teltschik et al., unpublished).

3.2. Low-density lipoprotein receptor-related protein 5 (LRP5)

As mentioned and shown above, the canonical Wnt pathway receptor LRP6 has been linked to ileal Crohn´s disease (CD) and, directly impacts on the expression of Paneth cell alpha-defensins. Besides that, canonical Wnt also influences gut mucosal proliferation and small intestinal Paneth cell maturation. Because of the important role of LRP6 in regulating Wnt target genes the co-expressed receptor LRP5 was analysed. Both receptors are co-expressed and have overlapping functions while also

taking on context dependent and specific roles. Therefore LRP5 mRNA expression in patients was studied, the co-receptors functional impact on Paneth cell defensins was analysed, and the distribution of SNPs were studied to see whether a genetic association with the ileal subtype of CD might exist.

3.2.1 mRNA data

LRP5 is constitutively expressed at the cell surface (Joiner et al. 2013) and is also involved in β-catenin dependent Wnt signal transduction. To examine if this receptor is also changed in Crohn's disease, differences in LRP5 mRNA expression were analysed by quantitative real- time PCR in the Stuttgart cohort. This comprises controls and patients suffering from L1, L2 and L3 CD. Additionally a pediatric cohort from Norway comprising controls and L1, L2 and L3 CD patients was studied.

3.2.1.1 LRP5 expression in the ileal CD is slightly decreased

The Stuttgart cohort comprises 28 healthy controls and 72 CD patients. The samples were sub-grouped according to the disease state and patients/controls with mutations in the pattern recognition receptor NOD2 as well as patients with a neoterminal ileum were excluded. MRNA levels of patients with solely ileal Crohn's disease (L1), Crohn's disease of the small intestine and the colon (L3) and patients with solely colonic involvement of disease (L2) in comparison to healthy controls were analysed. L1 patients showed slightly but not significantly decreased levels of LRP5 mRNA in comparison to controls and to patients suffering from L2 CD (figure 7, lower panel). No differences between inflamed and not inflamed tissue was observed (figure 7, upper panel). To confirm the observed effects, LRP5 expression was analysed in the cohort from Norway. This cohort consists of pediatric patients, where the genetic influence might be more pronounced. LRP5 mRNA expression levels in the mucosa of 32 non-IBD control children in comparison to children suffering from Crohn's disease (n=28) was analysed. Again, a small but not significant, decrease of LRP5 mRNA levels was observed in the ileal CD group (L1, n=2). Also no significant differences were observed in the colonic CD group (L2, n=5) and in ileocolonic disease (L1+L3, n=23), (figure 8).

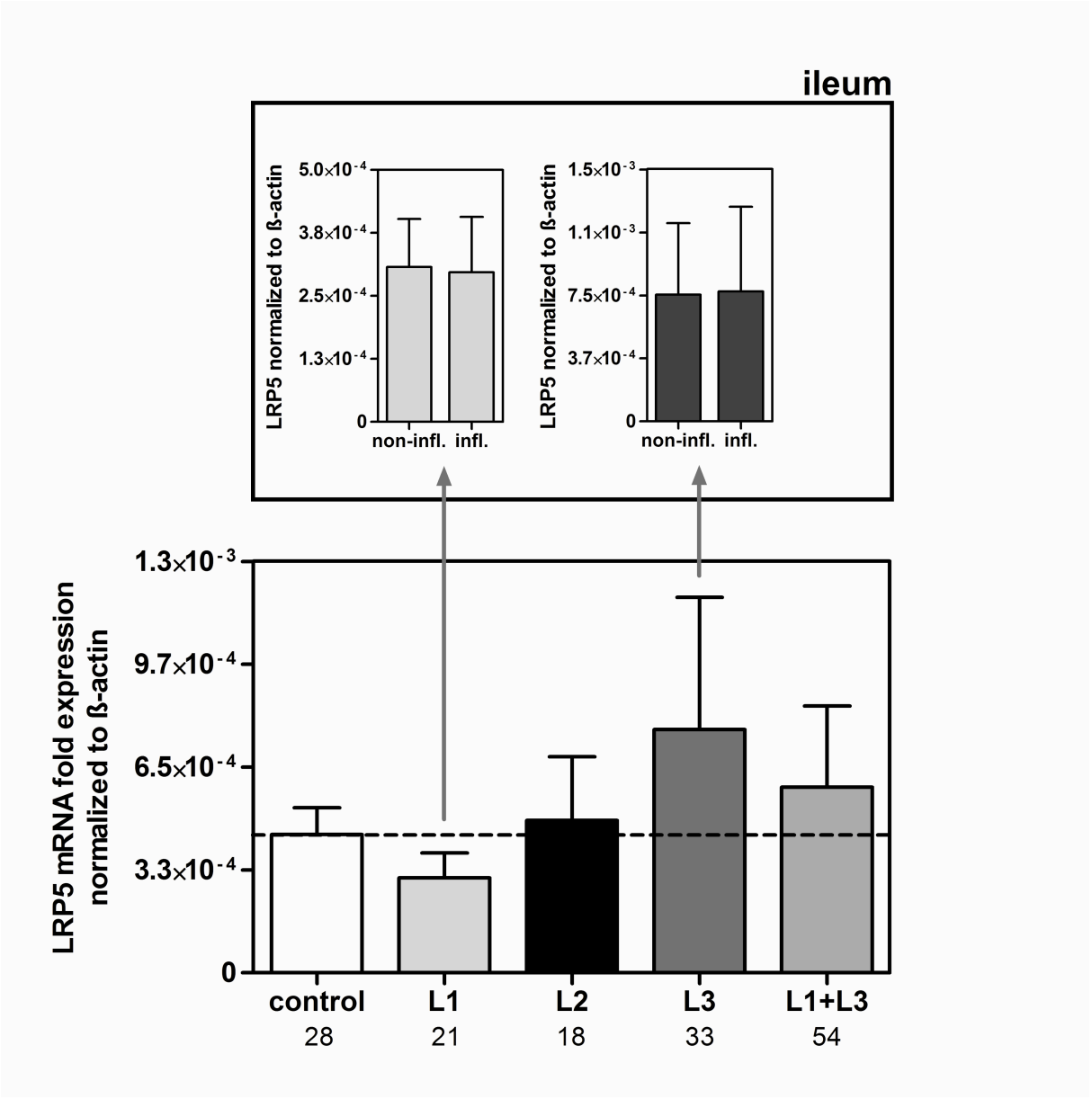

Figure 7: LRP5 mRNA expression in the ileum of Crohn´s disease patients from Stuttgart.
Slight but not significant decrease of LRP5 mRNA expression was observed in patients suffering from ileal Crohn's disease (L1) in comparison to the healthy control group and patients suffering from colonic Crohn's disease (L2, lower panel). No differences were seen between inflamed samples and non-inflamed samples (upper panel), (Teltschik et al., unpublished).

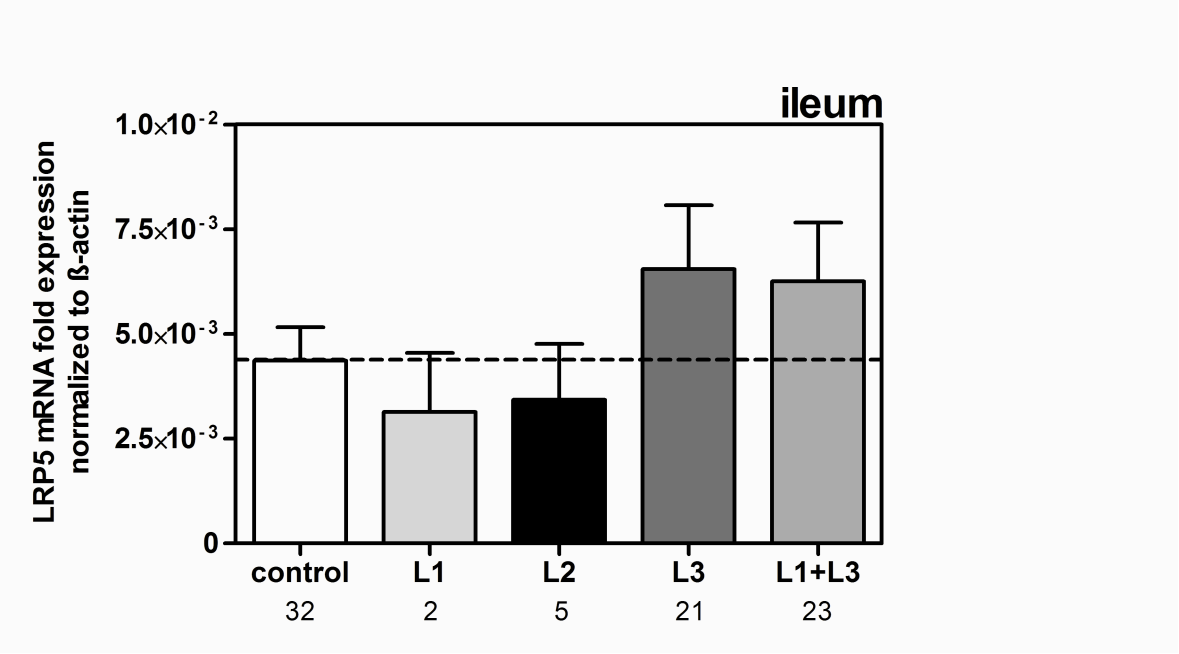

Figure 8: LRP5 expression in the ileal mucosa of pediatric Crohn's disease patients from Norway. No significant differences of LRP5 mRNA expression were observed in patients suffering from ileal Crohn's disease (L1, n=2) in comparison to a healthy non-IBD control group (n=32) and patients suffering from colonic Crohn's disease (L3, n=21), (Teltschik et al., unpublished).

3.2.1.2 HD-5 expression is significantly decreased in ileal and ileocolonic CD

As previously described, the regulation of α- defensin 5 (HD-5) is linked to the Wnt signaling pathway (Wehkamp et al. 2007; Koslowski et al. 2012; Beisner et al. 2014).

Measuring the mRNA expression revealed significantly decreased levels of HD-5 in patients with ileal CD (L1, p=0.0023) and patients with colonic CD (L3, p=0.0277), as well as in ileocolonic disease (L1+L3, p=0.0029) in comparison to L2 CD patients and healthy controls (figure 9). This decrease was independent of inflammation (data not shown). As LRP5 expression was not significantly chanced, this significant decrease of HD-5 indicates a mechanism not depending on LRP5 expression. Similar to the effects seen in the Stuttgart cohort, in the Norwegian cohort levels of HD-5 were significantly decreased in children suffering from ileocolonic Crohn's disease (L1+L3, p=0.0298, L3, p=0.0347) in comparison to children suffering from solely colonic disease (L2) and a non-IBD control group (data not shown) (Perminow et al. 2010). Due to the fact that LRP5 mRNA levels are unchanged, this mechanism also seems to be independent from LRP5 expression.

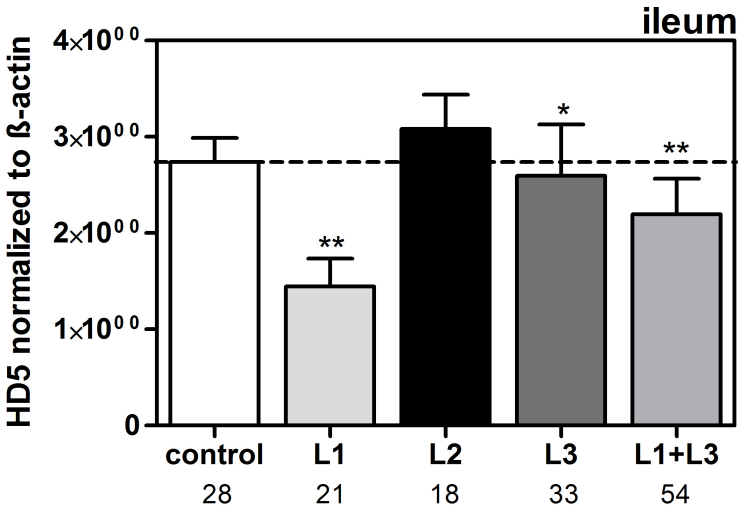

Figure 9: Expression of human α-defensin 5 (HD-5) in the ileal mucosa of CD patients from Stuttgart. MRNA expression of HD-5 is significantly decreased in patients suffering from ileal Crohn's disease (L1) as well as in patients with colonic and ileal involvement of disease (L3),* $p < 0.05$, ** $p < 0.01$ (Teltschik et al., unpublished).

3.2.1.3 LRP5 mRNA expression seems to be unchanged in colonic biopsies

Additionally the expression of LRP5 in mRNA isolated from colonic mucosal tissue from the Stuttgart cohort was analysed. No significant differences between patients with any subgroup of Crohn's disease and the control group were detected (figure 10). Again, also in the pediatric cohort from Norway the colonic tissue was analysed. Although no significant differences in LRP5 mRNA expression between patients with any subgroup of Crohn's disease and the control group (figure 11) was seen, a slight decrease in expression in all CD subgroups comparable to the slight decrease of LRP5 expression in the ileum in L1 patients was observed.

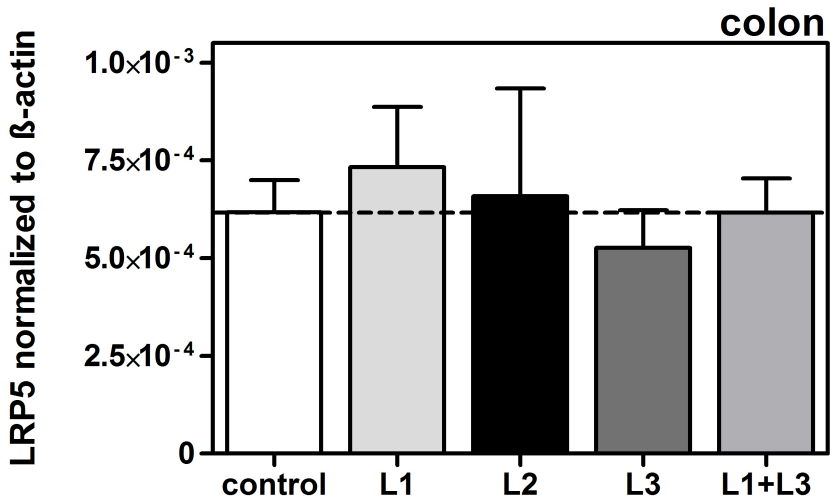

Figure 10: LRP5 mRNA expression in the colon of Crohn´s disease patients from Stuttgart.
No significant differences in LRP5 mRNA expression in patients suffering from ileal Crohn's disease (L1) in comparison to the healthy control group and patients suffering from colonic Crohn's disease (L2), (Teltschik et al., unpublished).

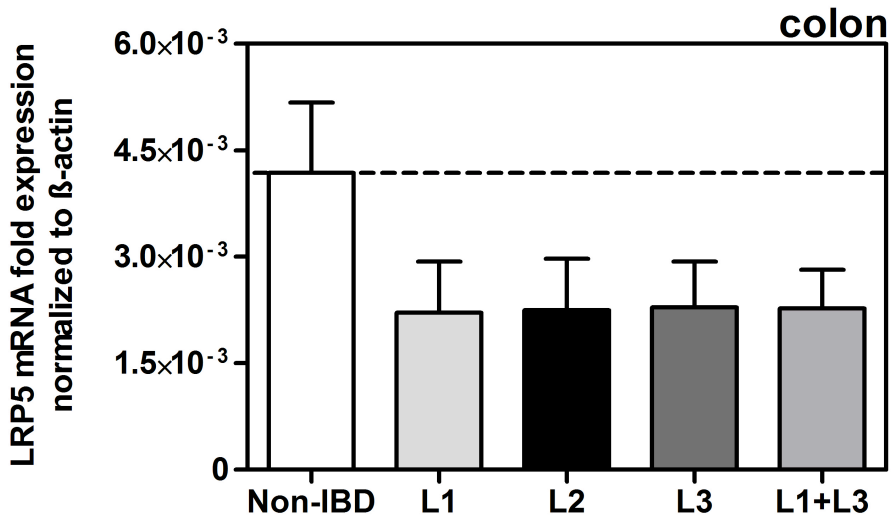

Figure 11: LRP5 expression in the colon of pediatric Crohn´s disease patients from Norway.
LRP5 is not significantly decreased in colonic tissue of patients suffering from ileal or colonic Crohn's disease (L1 n=2, L2 n=5, L3 n=20) in comparison to a healthy non-IBD control group (n=32), (Teltschik et al., unpublished).

3.2.2 LRP5 overexpression experiments in HEK-293 cells

To elucidate the function of LRP5 relating to its role in the activation of Wnt target genes HD-5 and HD-6, overexpression experiments in HEK-293 cells were performed. LRP6 and LRP5 have overlapping but also distinct functions and LRP6 is thought of having a stronger role in the activation of Wnt target genes (MacDonald et al. 2011). As shown above, LRP6 overexpression leads to activation of HD-5/-6. Therefore the LRP6 gene was knocked down with siRNA to study the role of LRP5 alone. Experiments were performed at least three times and transfections were carried out in triplicates. The displayed values represents averages with SEM indicated by error bars.

3.2.2.1 *In vitro* β-catenin dependent Wnt HD-5 target gene promoter activation seems to be independent of LRP5 expression levels.

To elucidate the role of LRP5 *in vitro*, human embryonic kidney cells (HEK-293 cells) were transfected with a LRP5 overexpression plasmid, harbouring the active form of LRP5. This overexpression was however not sufficient to transactivate the co-transfected HD-5 (figure 12, upper panel) or HD-6 (data not shown) promoter when compared to an empty plasmid or medium controls.

3.2.2.1.1 Additional stimulation with Wnt1 does not induce HD-5 promoter activity

To counteract a potentially low activity of LRP5 in comparison to LRP6, additional stimulation with Wnt1 was performed, which is a ligand known to interact with the LRPs (Bhat et al. 2007). Furthermore, intracellular LRP6 was also knocked-down with siRNA to avoid effects, which might allow a strong LRP6 function to level out any LRP5 mediated changes in activity (figure 12, lower panel). No significant induction of HD-5 promoter activity was observed after overexpression and additional Wnt1 stimulation of LRP5 and parallel knockdown of intracellular LRP6 in HEK-293 cells in comparison to cells transfected with a negative control siRNA and to unstimulated cells and also to a non-targeting control siRNA (figure 12, lower panel).

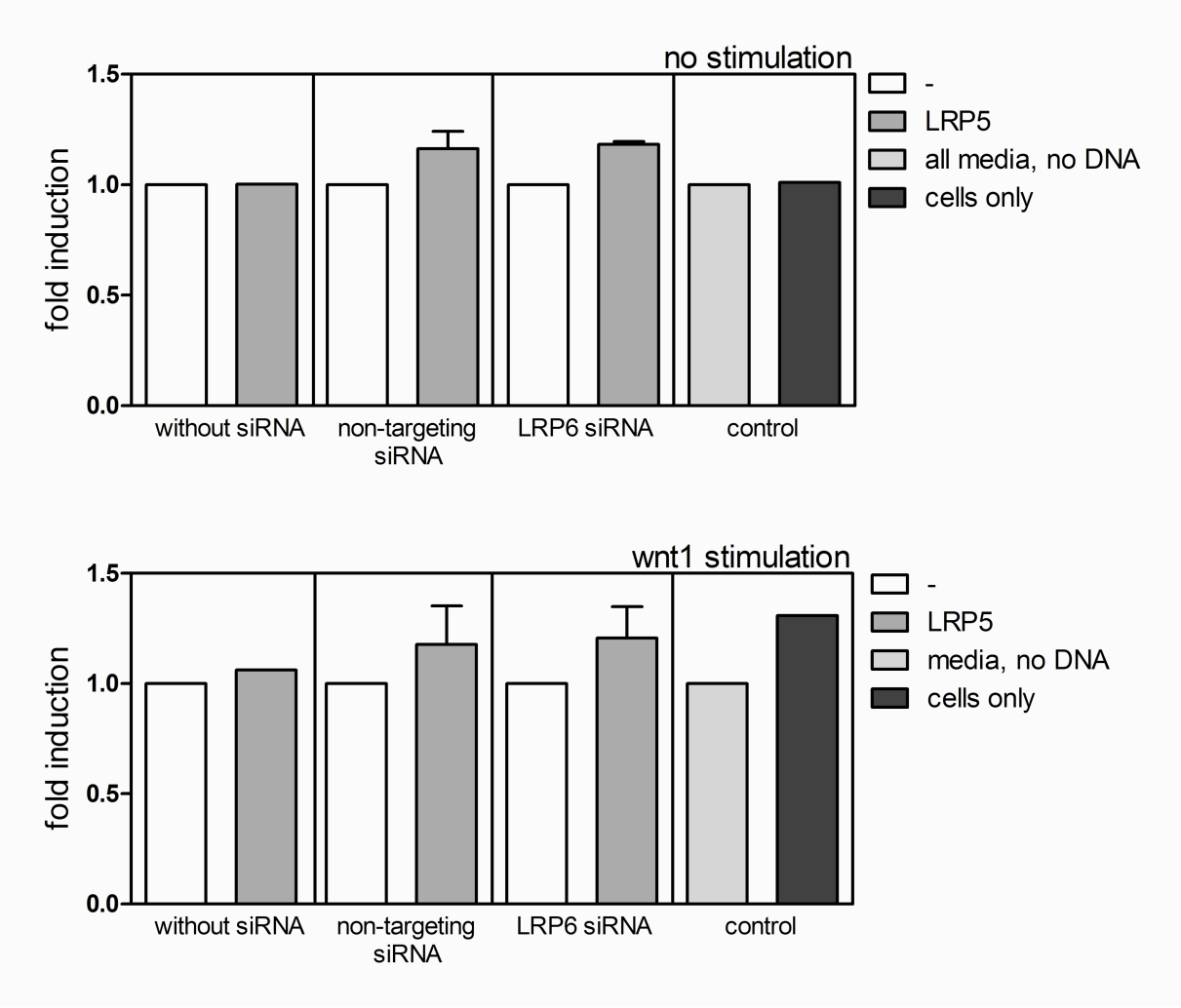

Figure 12: No significant transactivation of the HD-5 promoter activity via overexpression of LRP5 transfection plasmid and either no stimulation or stimulation with the LRP5 ligand Wnt1 in HEK-293 cells: The transcriptional activity of a luciferase reporter under the control of a 1kb HD-5 promoter was not significantly increased after LRP5 mediated activation of β-catenin dependent Wnt signaling. Knockdown of LRP6 to reveal effects of LRP5 which, could be overlain by LRP6, only very slightly increased the effects of LRP5 overexpression. Values are the average of triple determinations with the SEM indicated by error bars (Teltschik et al., unpublished).

3.2.2.1 *In vitro* β-catenin dependent Wnt TopFlash target gene promoter activation seems to be independent of LRP5 expression levels.

To test whether overexpression of LRP5 per se leads to stronger Wnt signaling activity, HEK-293 cells were transfected with the active LRP5 expression plasmid and the TopFlash plasmid, a luciferase reporter that contains multiple repeated copies of wild-type TCF/LEF-binding sites. TopFlash can consequently very effectively be induced via β-catenin-mediated transcriptional activation. LRP6 expression was knocked-down with siRNA and LRP5 signaling activity was either not stimulated, stimulated with Wnt1 or Wnt3a, which has also been shown to increase LRP5 activity before (Grumolato et al. 2010). Although slight upregulation after Wnt3a stimulation was observed, no significant transactivation of β-catenin dependent Wnt signaling via overexpression of LRP5 transfection plasmid and either no stimulation, stimulation by the LRP5 ligand Wnt1 or Wnt3a was observed (figure 13).

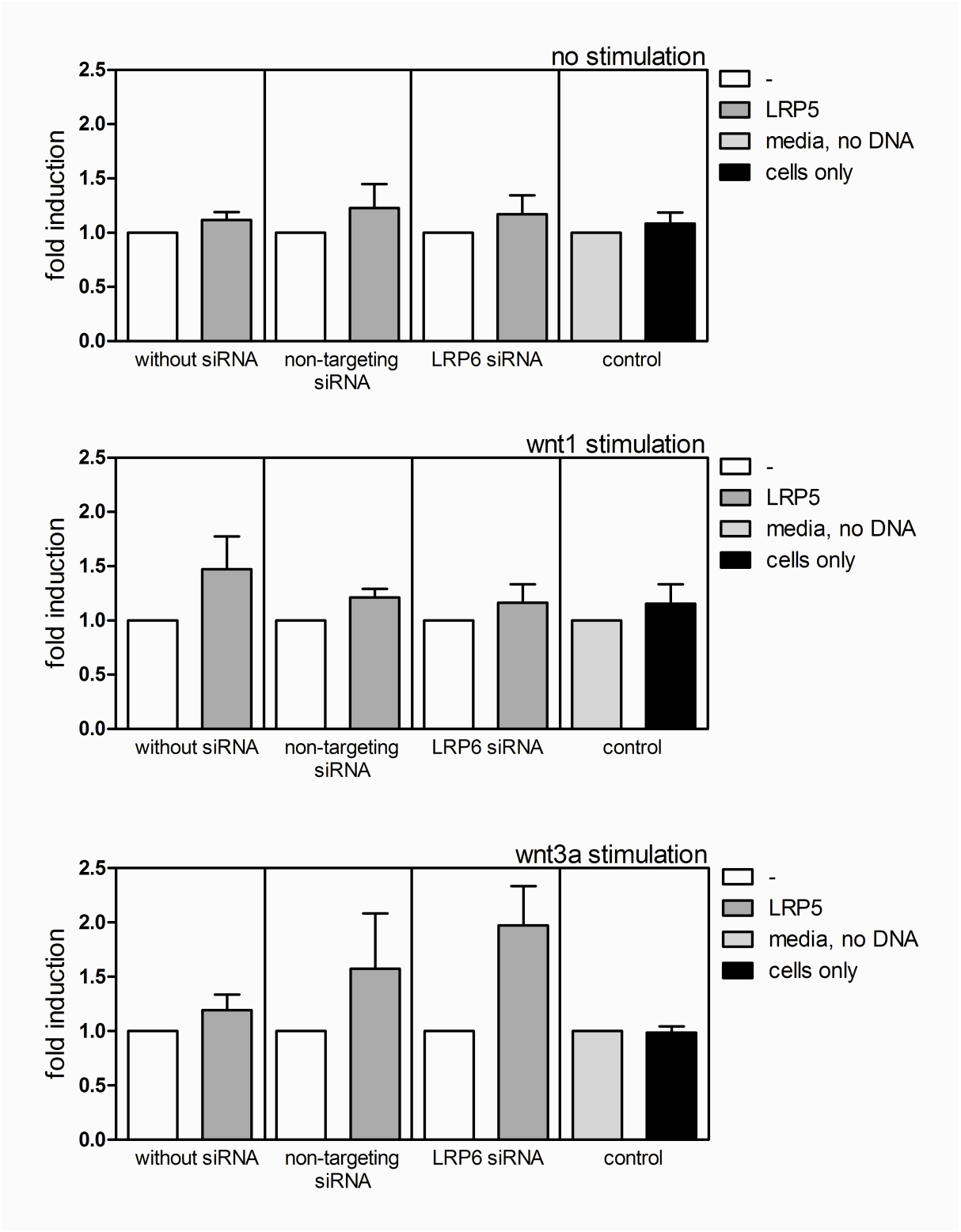

Figure 13: No significant transactivation of β-catenin dependent Wnt signaling via overexpression of LRP5 transfection plasmid and either no stimulation, stimulation with the LRP5 ligand wnt1 or wnt3a in HEK-293 cells: Overexpression of LRP5 in HEK-293 cells leads to an slightly but not significant activated β-catenin dependent Wnt signaling as monitored by TopFlash activity. Knockdown of LRP6 to reveal effects of LRP5, which could be overlaid by LRP6 did not or only very slightly (for stimulation with wnt3a) increased the effects of LRP5 overexpression. Values are the average of triple determinations with the SEM indicated by error bars (Teltschik et al., unpublished).

3.2.3 Genetic analysis of the candidate gene LRP5

As described (M. J. Koslowski et al. 2012) mutations in the LRP6 gene are associated with early onset Crohn´s disease. LRP6 and LRP5 are co-expressed receptors which often act together and are both needed to respond to some Wnt signals (Goel et al. 2012). Although a direct influence of LRP5 on HD- and -6 was not detected on mRNA level, Wnt is important for normal epithelial homeostasis (Fevr et al. 2007), which is disturbed in CD. Therefore a potential genetic association of LRP5 to Crohn´s disease was investigated. 45 SNPs in the LRP5 gene (figure 14) in three cohorts (Leuven, Stuttgart, Vienna) were analysed. The analysis included exonic, coding SNPs, which lead to an amino acid change (indicated in red), exonic synonymous SNPs, which do not lead to an amino acid change (indicated in green) and intronic SNPs, which are indicated in blue based on selection criteria as listed in "methods".

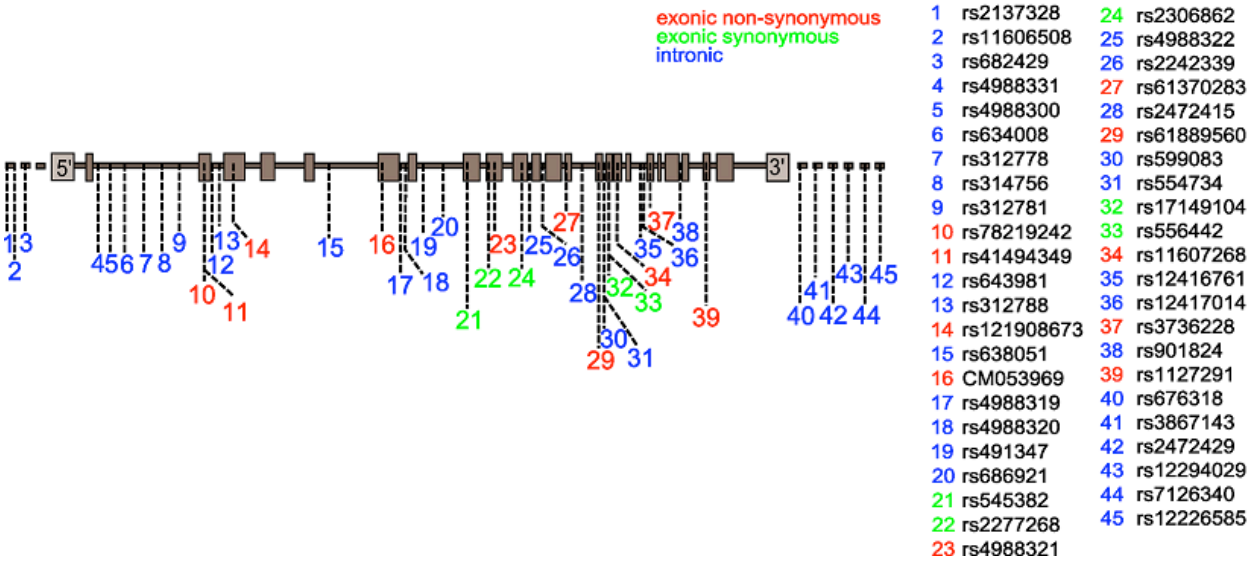

Figure 14: Schematic representation of the human LRP5 gene, indicating the relative location of the analysed single nucleotide polymorphisms (SNPs) that belong to five haploblocks (see figure 15), (Teltschik et al., unpublished).

The samples were sub-grouped according to gender, age, age of onset of disease (A1: 16 years or younger, A2: 17–40 years and A3: >40 years), behaviour of disease and involvement of either small intestine (L1), colon (L2), both small intestine and colon (L3) and additional involvement of the upper gastrointestinal tract (L4). Analysis of single cohorts showed very significant differences in the disease group in comparison to controls, as described below. But analysis of all three cohorts compensated the obvious differences between the analysed groups. This is due to often observed reversed results in one cohort in comparison to another (see

table 8). Table 8 shows three SNPs as examples. SNP1´s (examples in green) minor allele frequency (MAF) in the Leuven cohort is 7.87% in the control group and 14.71% in the group with patients suffering from early onset of disease. In the Vienna cohort it is 13.94% in the control group and 4.17% on the early onset group, so the results are reversed in the two different cohorts. In the third cohort (Stuttgart) the trend is the same than in the Vienna cohort, but the MAFs are much lower. SNP2 (examples in orange) has a MAF of 29.55% in the Leuven control group and a MAF of 36.41% in the ileal CD group. The Vienna control group has a MAF of 41.63% in comparison to 30.81% for patients suffering from ileal CD and in the Stuttgart cohort 29.14% MAF in the control group and 30.56% in the ileal CD group. These examples show very nicely the problems analysing the different cohorts due to the uneven distribution of the analysed SNPs in the different cohorts. This highlights a high level of heterogeneity in the population, which might be due to the fact that many SNPs might have some minor benefit or drawback or show associations with certain phenotypes, which cannot be accounted for. LRP5 deficiencies are indeed linked to many other disease for example increased fracture risk, osteoporosis (Urano et al. 2009; Korvala et al. 2012; Xiong et al. 2007), and also familial exudative vitreoretinopathy (Jiao et al. 2004), but some SNPs are on the other hand also associated with higher bone mineral density (BMD). More data depending disease status and phenotype regarding BMD are not available, so it was not possible to exclude all the samples, which could lead to a bias in the analysis. For overall allele frequencies see appendix.

Table 8: Exemplarily the minor allele frequencies of 3 SNPs to demonstrate the observed differences in the analysed cohorts (MAF: minor allele frequency, Divert: diverticulitis, C. Indet.: colitis indeterminata, CD: Crohn's disease).

LEUVEN MAFs	rs314756 SNP 1 intronic	rs676318 SNP 2 intronic	rs556442 SNP 3 exonic	**VIENNA** MAFs	rs314756 SNP 1 intronic	rs676318 SNP 2 intronic	rs556442 SNP 3 exonic	**STUTTGART** MAFs	rs314756 SNP 1 intronic	rs676318 SNP 2 intronic	rs556442 SNP 3 exonic
Controls	7.87%	5.95%	29.55%	Controls	13.94%	9.24%	41.63%	Controls	8.45%	6.74%	29.14%
Divert.	23.84%	10.56%	49.40%					C. indet.	6.25%	10.42%	29.17%
IBD	9.26%	8.49%	32.47%	IBD	6.32%	8.31%	31.86%	IBD	8.38%	6.13%	32.49%
UC	12.50%	5.49%	24.73%	UC	6.99%	10.33%	35.16%	UC	7.30%	6.67%	33.46%
CD	8.18%	9.49%	35.09%	CD	5.85%	6.90%	29.54%	CD	9.14%	5.76%	31.82%
male	10.23%	8.82%	35.38%	male	5.04%	8.62%	29.57%	male	6.77%	6.45%	35.05%
female	8.53%	8.81%	31.37%	female	6.44%	4.55%	26.54%	female	11.34%	5.32%	29.90%
A1	14.71%	11.76%	35.29%	A1	4.17%	6.25%	27.08%	A1	3.03%	1.52%	25.76%
A2	8.03%	8.99%	31.25%	A2	5.66%	6.97%	29.85%	A2	9.92%	6.47%	34.02%
A3	11.64%	4.79%	30.67%	A3	8.62%	6.90%	29.31%	A3	17.39%	11.36%	39.13%
B1	7.14%	11.43%	36.96%	B1	3.30%	6.31%	27.18%	B1	6.34%	7.14%	31.94%
B2	7.02%	12.07%	31.03%	B2	12.50%	3.13%	25.00%	B2	13.64%	5.56%	29.10%
B3	8.47%	7.69%	34.32%	B3	5.09%	8.41%	33.33%	B3	5.77%	8.00%	39.42%
L4*	7.14%	9.52%	38.10%	L4*	3.97%	13.71%	30.65%	L4*	4.55%	13.64%	54.55%
ileal CD	8.80%	9.95%	36.41%	ileal CD	5.39%	6.44%	30.81%	ileal CD	10.06%	5.88%	30.56%
male	9.68%	9.68%	39.13%	male	4.35%	8.33%	32.39%	male	7.69%	6.67%	33.13%
female	8.13%	10.16%	34.40%	female	6.00%	3.50%	26.02%	female	12.34%	5.41%	29.49%
A1	14.00%	18.00%	48.00%	A1	5.26%	5.26%	26.32%	A1	3.85%	1.92%	27.78%
A2	7.88%	8.90%	34.25%	A2	5.99%	6.44%	31.90%	A2	10.50%	6.84%	33.82%
A3	8.75%	10.00%	40.24%	A3	10.00%	7.50%	37.50%	A3	20.59%	9.38%	35.29%
B1	10.23%	14.77%	46.51%	B1	2.24%	6.82%	32.03%	B1	6.12%	4.17%	26.00%
B2	7.55%	12.96%	32.41%	B2	10.23%	3.41%	25.00%	B2	14.17%	6.14%	28.69%
B3	8.80%	7.01%	34.40%	B3	5.43%	6.59%	32.02%	B3	7.89%	9.72%	39.74%
L4*	7.14%	9.52%	38.10%	L4*	3.97%	13.71%	30.65%	L4*	4.55%	13.64%	54.55%

3.2.3.1 Haplotypes

To see whether a certain combination of SNP variants might predispose to CD, haplotype blocks and linkage disequilibria were calculated with Haploview. Analysis of haplotypes for the 45 analysed SNPs in the three cohorts revealed 5 haploblocks (figure 15). Neither allele combination within these blocks was however significantly associated with small intestinal CD as the differences between patients who suffer from ileal CD and a healthy control group were not significant (table 9). Association of haploblocks in haplotype analysis for all cohorts are listed in table 10.

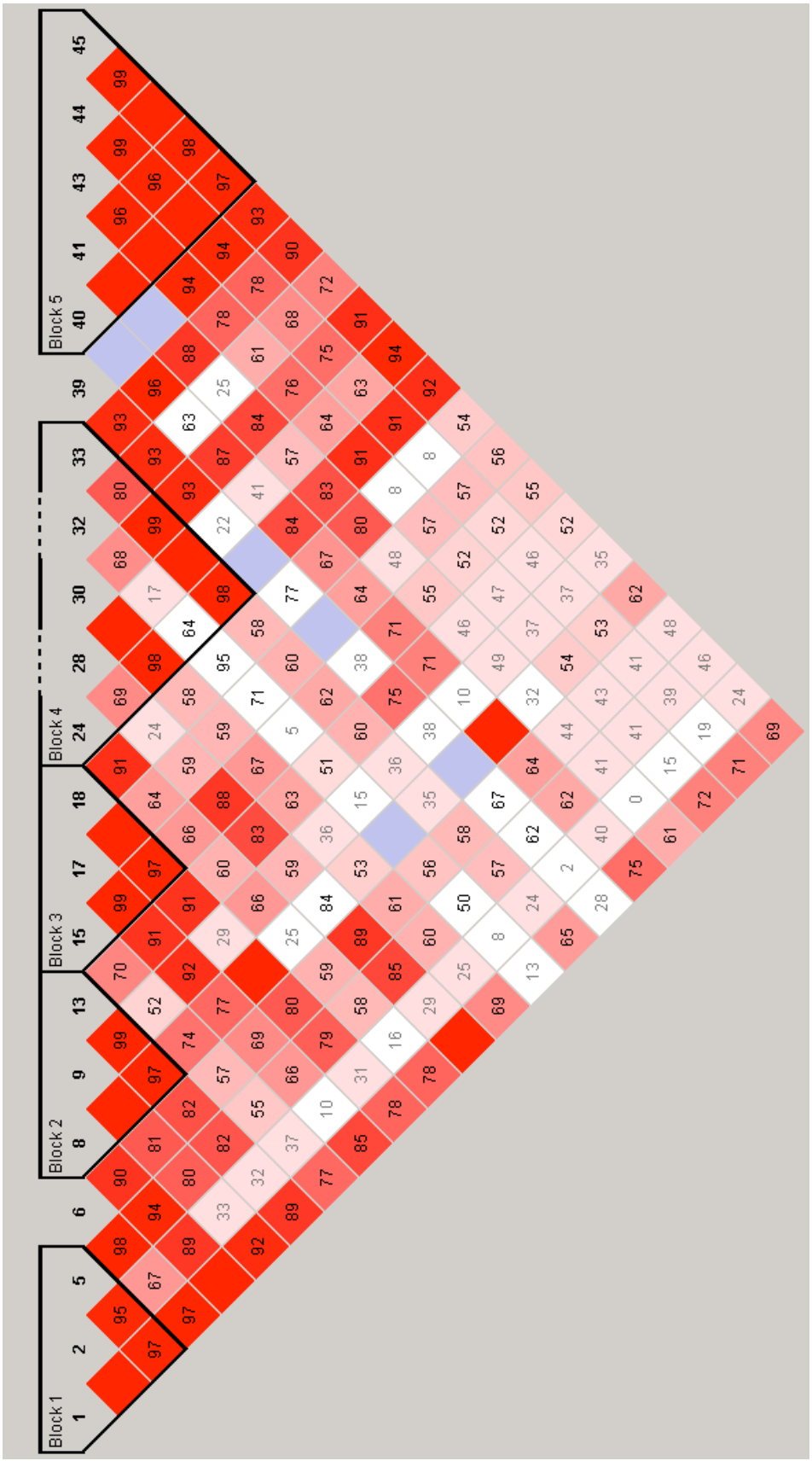

Figure 15: Haplotype blocks and linkage disequilibria were calculated with Haploview. Five haploblocks were found. But no significant differences between patients who suffer from ileal Crohn's disease and a healthy control group were found. D' and LOD (log of the likelihood odds ratio) values were used for selection of LD colour scheme in the discovery phase. None of the five blocks found in LRP5 was significant (Teltschik et al., unpublished).

Table 9: Association of haploblocks in haplotype analysis for all cohorts
(cases and controls in ileal CD)

haplotype	frequency	case | control ratios	chi square	p-value
BLOCK 1				
CCT	0.357	0.349 | 0.359	0.279	0.5975
CCG	0.312	0.311 | 0.312	0.003	0.9593
TCG	0.194	0.207 | 0.189	1.263	0.2612
CTT	0.131	0.127 | 0.133	0.221	0.6384
BLOCK 2				
ATT	0.587	0.602 | 0.582	0.984	0.3212
ACG	0.210	0.197 | 0.215	1.150	0.2836
ATG	0.121	0.132 | 0.116	1.448	0.2289
GCG	0.081	0.069 | 0.085	2.253	0.1334
BLOCK 3				
AGG	0.605	0.610 | 0.604	0.088	0.7673
GAG	0.157	0.160 | 0.156	0.072	0.7882
GGA	0.150	0.157 | 0.148	0.381	0.537
GGG	0.085	0.071 | 0.091	3.084	0.0791
BLOCK 4				
CTA	0.670	0.690 | 0.662	2.249	0.1337
CGG	0.168	0.165 | 0.169	0.076	0.783
TGG	0.149	0.136 | 0.154	1.580	0.2087
BLOCK 5				
TAACT	0.649	0.667 | 0.642	1.701	0.1921
TGCTA	0.136	0.115 | 0.144	4.134	0.042
TACTA	0.126	0.137 | 0.121	1.339	0.2472
CAACA	0.082	0.073 | 0.085	1.306	0.2532

Table 10: Association of SNPs in cases and controls for all cohorts in ileal CD

SNP number	rs number	assoc. allele	case | control ratios	chi-square	p-value
1	rs2137328	T	0.209 | 0.192	1.086	0.2973
2	rs11606508	C	0.869 | 0.863	0.2	0.6551
5	rs4988300	G	0.521 | 0.504	0.778	0.3777
6	rs634008	T	0.544 | 0.531	0.437	0.5088
8	rs314756	A	0.931 | 0.913	2.653	0.1034
9	rs312781	T	0.733 | 0.701	3.131	0.0768
13	rs312788	T	0.602 | 0.587	0.546	0.4601
15	rs638051	A	0.613 | 0.601	0.381	0.537
17	rs4988319	A	0.157 | 0.147	0.52	0.4707
18	rs4988320	A	0.157 | 0.147	0.535	0.4645
24	rs2306862	C	0.863 | 0.848	1.087	0.2972
28	rs2472415	T	0.049 | 0.029	6.008	0.0142
30	rs599083	T	0.701 | 0.681	1.098	0.2947

32	rs17149104	T	0.019 | 0.017	0.261	0.6098
33	rs556442	A	0.689 | 0.661	2.14	0.1435
39	rs1127291	T	0.015 | 0.009	2.246	0.134
40	rs676318	T	0.925 | 0.916	0.614	0.4332
41	rs3867143	A	0.887 | 0.855	4.916	0.0266
43	rs12294029	A	0.748 | 0.733	0.671	0.4127
44	rs7126340	C	0.750 | 0.738	0.429	0.5124
45	rs12226585	T	0.669 | 0.646	1.444	0.2295

3.3 T cell transcription factor 1 (TCF-1)

Parts of these results have previously been published in:

Intestinal Stem Cells in GI Physiology and Disease TCF-1-mediated Wnt signaling regulates Paneth cell innate immune defense effectors HD-5 and -6: implications for Crohn's disease

Julia Beisner, Zora Teltschik, Maureen J. Ostaff, Machteld M. Tiemessen, Frank J. T. Staal, Guoxing Wang, Michael Gersemann, Gori Perminow, Morten H. Vatn, Matthias Schwab, Eduard F. Stange, Wehkamp. Am J Physiol Gastrointest Liver Physiol. 2014 Sep 1;307(5):G487-98. doi: 10.1152/ajpgi.00347.2013.Epub 2014 Jul 3.

Author contributions:

J.B., Z.T., M.J.O., G.W., E.F.S., and J.W. conception and design of research; J.B., Z.T., M.J.O., M.M.T., F.J.S., G.W., M.G., and G.P. performed experiments; J.B., Z.T., M.J.O., M.G., and J.W. analyzed data; J.B., Z.T., M.J.O., M.H.V., M.S., E.F.S., and J.W. interpreted results of experiments; J.B., Z.T., and M.J.O. prepared figures; J.B. and J.W. drafted manuscript; J.B., Z.T., M.J.O., M.M.T., F.J.S., G.P., M.H.V., M.S., E.F.S., and J.W. edited and revised manuscript; J.B., Z.T., M.J.O., M.M.T., F.J.S., G.W., M.G., G.P., M.H.V., M.S., E.F.S., and J.W. approved final version of manuscript.

3.3.1 IHC staining reveals TCF-1 expression at the bottom of the crypts

Since our group could previously show that a decrease of Paneth cell α-defensins in patients with ileal CD is partially caused by impaired TCF-4 function (Jan Wehkamp et al. 2007; M. J. Koslowski et al. 2009), TCF-1 (also known as TCF-7) as another Wnt factor was analysed in the same context. TCF-1 protein in the small intestine was immunohistochemical stained to determine the expression pattern. The used antibody only detects the active isoform and not the inactive isoforms lacking the amino-terminal β-catenin binding domain. The active isoform is expressed predominantly at the bottom of the intestinal crypts in small intestinal cells (figure 16, left panel). This is the side where also the α-defensin producing Paneth cells are located. A corresponding section was phloxine-tartrazine stained; this shows the secretory granules in Paneth cells in pink (figure 16, right panel). As clearly seen, cells expressing the active TCF-1 colocalize with the stained Paneth cells. According to the important role of Wnt epithelial proliferation active TCF-1 was also detected above Paneth cells in epithelial progenitor cells as well as in some infiltrating gut lymphocytes.

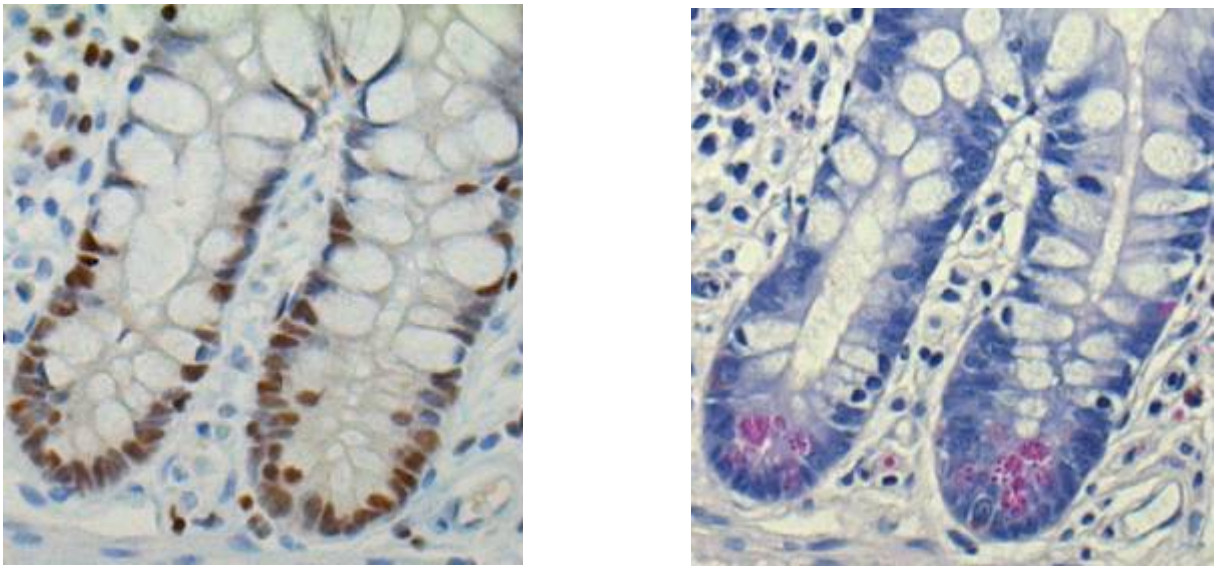

Figure 16: Immunohistochemical staining of the active TCF-1 isoform in small intestinal crypts (left panel) and phloxine tartrazine staining of human Paneth cell granules in the consecutive section (right panel), (Beisner, Teltschik et al., 2014).

3.3.2 TCF-1 expression is significantly decreased in ileal and ileocolonic CD

To study differences in mRNA expression of the transcription factor TCF-1 and its active isoform 1 it was performed quantitative real-time PCR in the Stuttgart cohort, comprising controls and patients suffering from L1, L2 and L3 Crohn's disease and a pediatric cohort from Norway comprising non-IBD controls and children suffering from L1, L2 and L3 CD.

In the Stuttgart cohort the expression of TCF-1 in the ileal mucosa tissue is significantly decreased in patients with ileal Crohn's disease (L1) as well as in patients with ileocolonic involvement of disease (L3) in comparison to patients with colonic CD (L3) and to the healthy control group (figure 17, A). In the Norwegian cohort, similar effects were detected. TCF-1 mRNA expression in biopsies isolated from the small intestine was significantly reduced ($p=0.0007$) in patients suffering from CD with ileal involvement (L1 and L3) in comparison to non-IBD controls and to patients suffering solely from colonic disease (figure 18, A). The latter showed only very slightly decreased levels of TCF-1.

In the Stuttgart cohort the mRNA expression of TCF-1 does not show any significant differences in the analysed groups in colonic mucosa tissue (figure 17, B).

The TCF-1 gene can generate full-length activating isoforms as well as dominant negative isoforms by alternative promoter usage. A real-time PCR assay was used, which only detects the active TCF-1 mRNA isoform 1 containing the β-catenin binding domain and found similar results then for general TCF-1. Patients with ileal and ileocolonic CD had significantly decreased levels of TCF-1 isoform 1 in the small intestine in comparison to colonic CD patients and to the control group (figure 17, C). Also the active TCF-1 isoform 1 mRNA in the Norwegian cohort was significantly reduced ($p=0.0008$) in L1 and L3 CD patients in comparison to the non-IBD control group and children suffering from colonic CD (figure 18, B). Again, the expression in the colonic biopsies revealed no significant differences, despite similar trends in the Stuttgart cohort (figure 17, D).

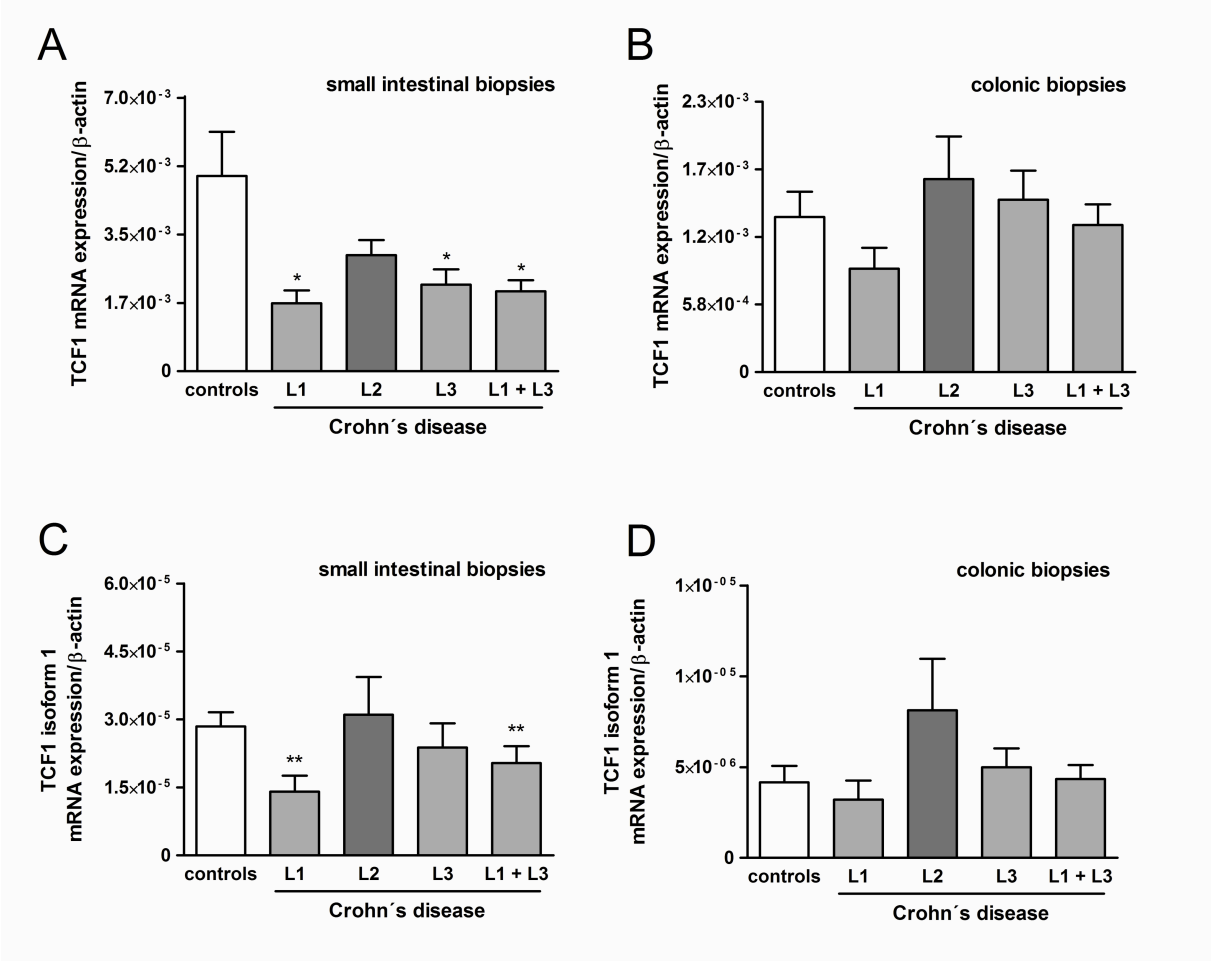

Figure 17: TCF-1 and active TCF-1 isoform expression in the ileal and colonic mucosa of healthy controls and patients with Crohn's disease from Stuttgart. TCF-1 (A, B) and active TCF-1 isoform (C, D) mRNA expression were quantified by LightCycler real-time reverse transcriptase PCR in small intestinal and colonic biopsies from a control group (n=27), Crohn's disease (CD) patients with ileal (L1, n=14), colonic (L2, n=19) and ileocolonic phenotype (L3, n=26) at diagnosis,* p <0.05, ** p < 0.01 (Beisner, Teltschik et al., 2014).

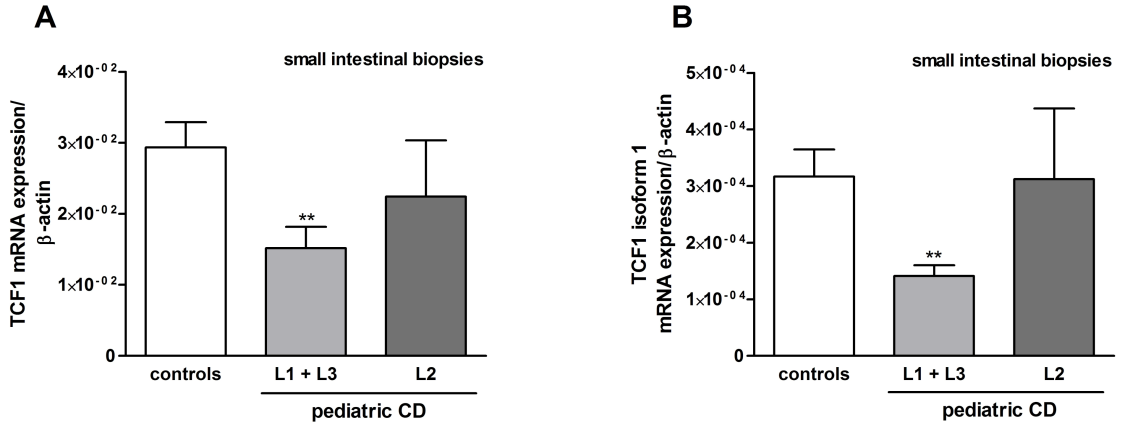

Figure 18: Expression of TCF-1 in the ileal mucosa of paediatric CD patients from Norway. Paediatric patients with ileal Crohn's disease (L1 + L3, n=22) show significantly decreased levels of TCF-1 (p=0.0007) (A) and active TCF-1 isoform 1 (B) in comparison to patients suffering from colonic Crohn's disease (L2, n=6) and non-IBD controls (n=29), ** p < 0.01 (Beisner, Teltschik et al., 2014).

3.3.3 TCF-1 knockout mice

To further study the functional role of TCF-1 *in vivo,* mRNA expression levels of different defensins, extracted from the middle part of the small intestine (jejunum) of TCF-1 knockout mice (with a C57BL6 background) were analysed by quantitative real- time PCR. To confirm the TCF-1 knockout TCF-1 expression on mRNA level was analysed. In heterozygous knockout mice the levels of TCF-1 were 2-fold lower than in wild type mice, in homozygous knockout mice the levels of TCF-1 were only very low residual, non-functional levels of TCF-1 detectable (data not shown). This is consistent with former data and relates to the fact that the knockout only affects parts of the gene as described in (F. J. Staal et al. 2001).

Further the expression of Defa21, which is a C57BL6 strain specific analogue to cryptdin 4 was measured (Shanahan, Tanabe, and Ouellette 2011). Heterozygous knockout mice show slightly reduced levels of this cryptdin in comparison to wild type mice, homozygous TCF-1 knockout mice showed even stronger, significantly reduced expression (figure 19, A). Additionally the expression of Defa24, lysozyme and cryptdin-related sequence-1C (CRS-1C) showed similar but not significant trends (figure 19, B, C, D). Analysis of the proximal and the distal part of the small intestine did not consistently show the same trends. To get a better overview of all cryptdins, a pan cryptdin assay (Shanahan et al. 2010) was performed to asses total cryptdin mRNA levels in the duodenum, the jejunum and the ileum of TCF-1 knockout mice. Pan cryptdin expression in the jejunum was significantly decreased in homozygous TCF-1 knockout mice in comparison to wild type mice (figure 20, A, B, C). These results are corresponding to the mentioned results for defa21. Further the expression of Reg3γ, the mouse analogue to the human Reg3α, which is one of the best-studied bactericidal lectins was analysed. In the duodenum in the jejunum, but not in the ileum, significantly increased expression in the TCF-1 knockout mice was found (figure 20, D, E, F).

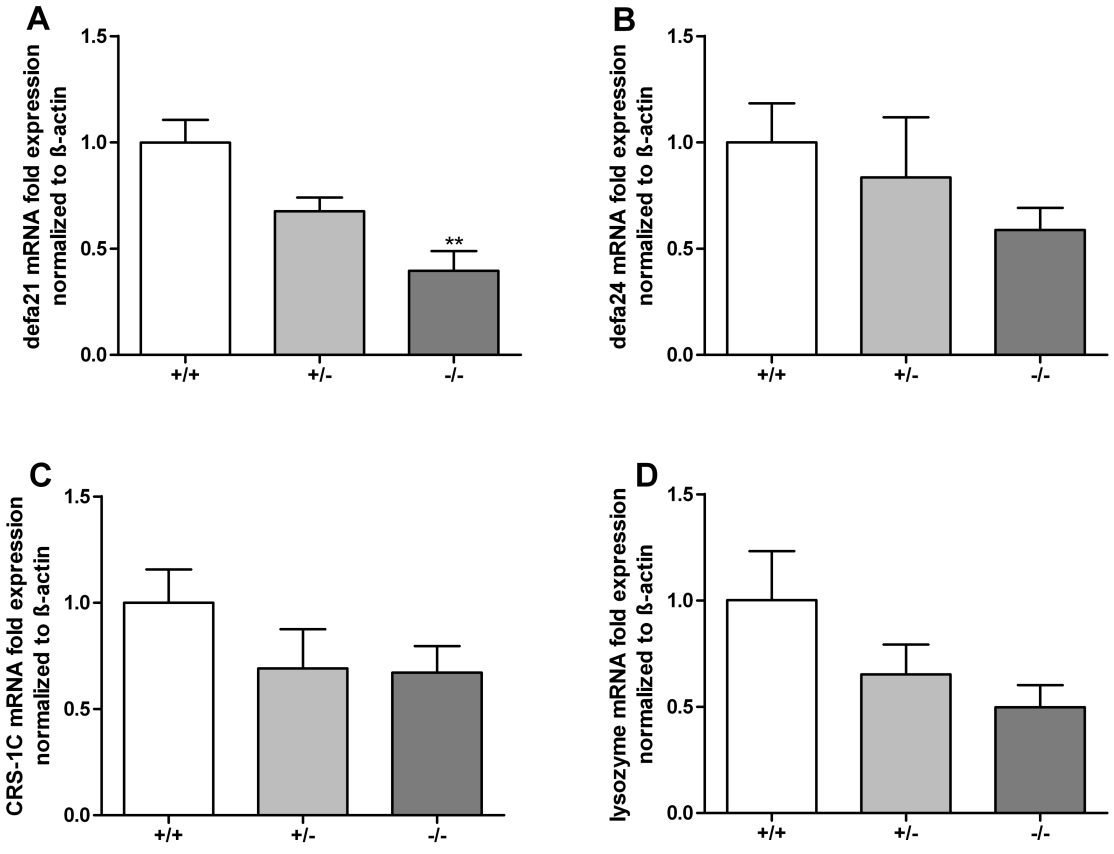

Figure 19: Expression of Paneth cell gene products in the small intestine of Tcf-1 1 +/+, +/- and -/- mice. mRNA expression levels of defa24 (A), defa21 (B), lysozyme (C) and cryptdin-related-sequence 1c (CRS1C) were quantified by LightCycler real-time reverse transcriptase PCR in the middle part of small intestinal mouse tissues of Tcf-1 +/+ (n=6), +/- (n=6) and -/- mice (n=6) . ** $p < 0.01$ (Beisner, Teltschik et al., 2014).

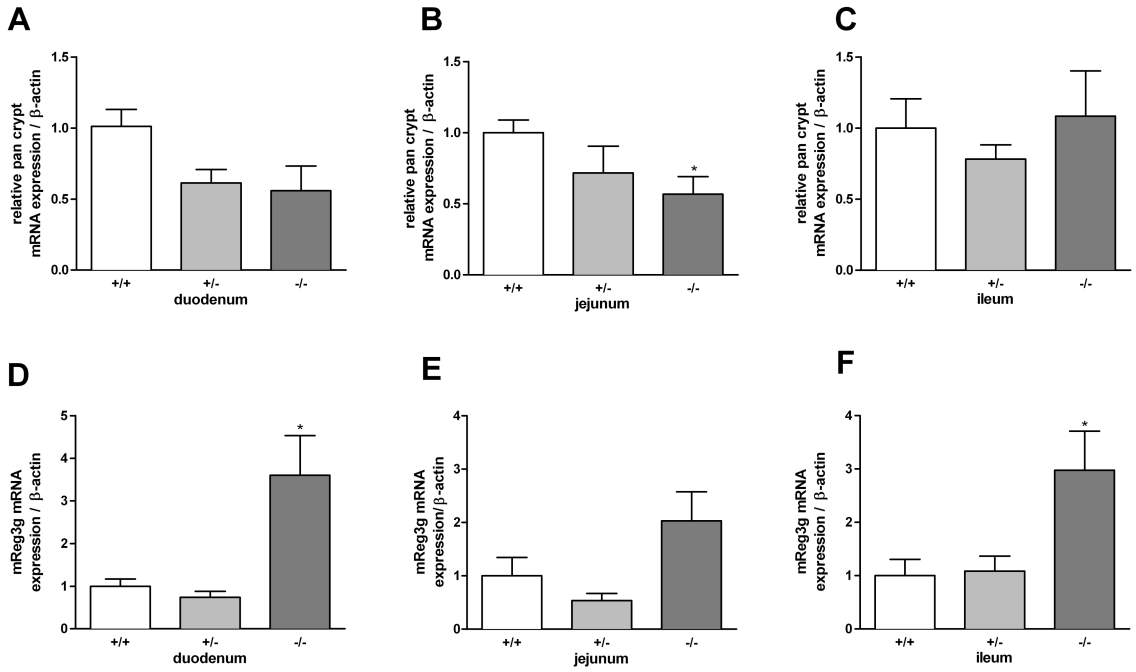

Figure 20: Expression of total Paneth cell α-defensins (pan cryptdin) and Reg3γ in the small intestine of of Tcf-1 +/+, +/- and -/- mice. mRNA expression levels of total cryptdins (A, B, C) and Reg3γ (D, E, F) in the tissue from the duodenum, jejunum and ileum from Tcf-1 +/+, +/- and -/- mice (each n=6), * $p < 0.05$ (Beisner, Teltschik et al., 2014) .

3.4 Functional relevance of antimicrobial defence regarding bacterial translocation in two animal models

Parts of these results have previously been published in:

Intestinal bacterial translocation in rats with cirrhosis is related to compromised Paneth cell antimicrobial host defense.

Teltschik Z1, Wiest R, Beisner J, Nuding S, Hofmann C, Schoelmerich J, Bevins CL, Stange EF, Wehkamp J. Hepatology. 2012 Apr;55(4):1154-63. doi: 10.1002/hep.24789. Epub 2012 Feb 15.

Author contributions:

Conceived and designed the experiments: ZT RW JB SN JW. Performed the experiments: ZT RW SN. Analyzed the data: ZT RW JB SN JW. Contributed reagents/materials/analysis tools: RW SN CH JS CLB EFS. Wrote the paper: ZT RW JB EFS JW. Obtained plasmids and performed pretests: GW. Involved in discussion of methods and data: ZT RW JB SN EFS CLB JW.

Since the study of antimicrobial function in the human *in vivo* situation provides various challenges and proves to be rather limited to descriptive data, it was aimed to further investigate the role of defensins and other antimicrobials in animal models. One event that is linked to impaired barrier function and therefore potentially to changes in antimicrobial defence, is the translocation of bacteria over the gastrointestinal mucosa. Mucosal adherent bacteria are seen in IBD and are likely linked to defective expression of antimicrobial peptides (Y. Liu et al. 1995; Darfeuille-Michaud et al. 2004; Swidsinski et al. 2005). In liver cirrhosis, another devastating gastrointestinal disorder, bacterial translocation (BT) has been implicated as a pathogenesis-promoting event. If and how antimicrobial defense at the intestinal barrier plays into a gut liver axis regarding bacterial translocation and the onset or maintenance of cirrhosis has yet to be studied.

3.4.1 Rats with liver cirrhosis and rats with portal vein ligation

To better understand the mechanisms, which could lead to deficiencies in antimicrobial defence in the intestine, two different rat models were analysed. From former studies it is known that patients with liver cirrhosis (LC) suffer from severe inflammations and sepsis, which is mostly caused by bacteria originating from the normal gut microbiota (Wiest and Garcia-Tsao 2005; Steffen, Berg, and Deitch 1988). The liver cirrhosis rat model comprised rats with a CCl_4 induced liver cirrhosis. The portal vein ligation (PVL) model comprised rats with a portal vein ligation, imitating the congestion of blood, which is typically observed in liver cirrhosis due to destroyed liver tissue, but without the cirrhosis. This model was used to exclude the possibility that the observed changes in the LC model are only due to liver cirrhosis- induced portal hypertension.

3.4.1.1 Rat model with liver cirrhosis

Rats in this model were exposed to CCl_4 (inhalation) along with phenobarbital in the drinking water (Wiest et al. 2003). After 12 to 16 weeks this approach induces micronodular liver cirrhosis with ascites.

3.4.1.2 Intestinal bacterial translocation in a rat liver cirrhosis model

BT was visualized with green fluorescent protein (GFP) labelled *E. coli*. To obtain this labelled *E. coli* a clinical isolate was transformed with a high-copy plasmid pCU18-GFP, carrying a modified gfp-gene. 10^8 labelled *E. coli* were administered to the rats and six hours later MLN and ascites fluid were harvested and cultured. GFP-

marked *E. coli* bacteria were found along the gastrointestinal (GI) tract in the stool but also in mesenteric lymph nodes, indicating a translocation of these bacteria (figure 21, C). This work was performed in cooperation with Rainer Wiest from the University Hospital Regensburg.

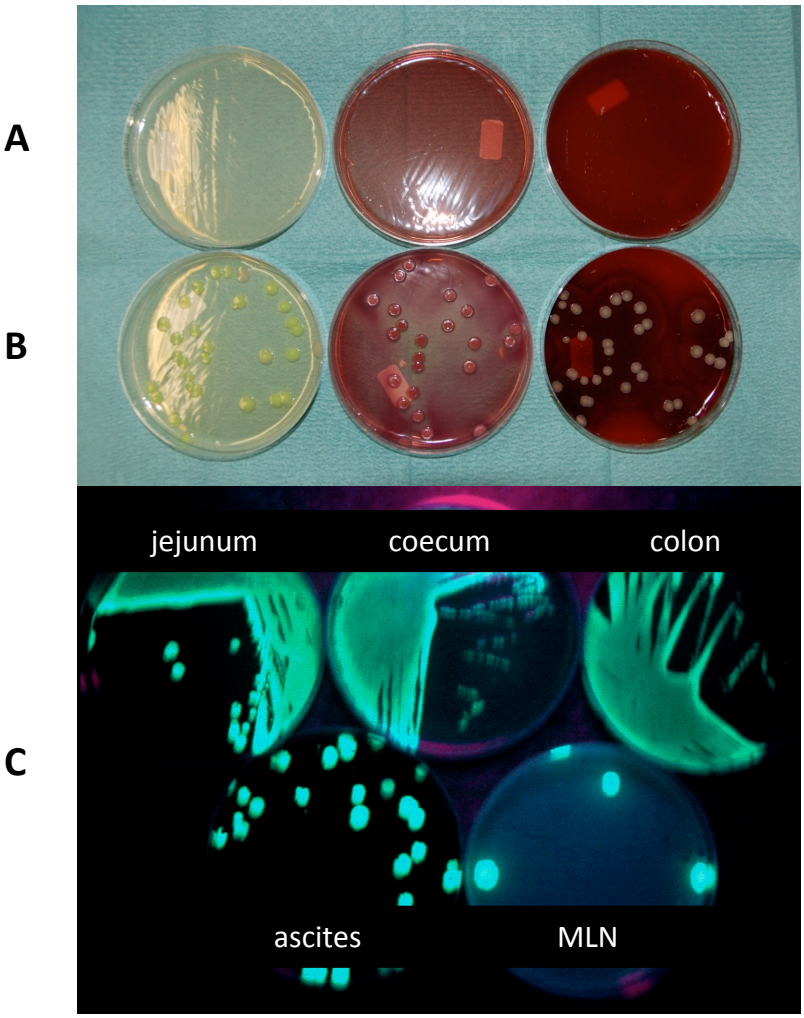

Figure 21: Representative bacteria culture of mesenteric lymph nodes (MLN) from rats with cirrhosis and ascites. A: Animal without bacterial translocation (BT): sterile plates after 48 hours of incubation. B: Animal with BT: endogenous commensal *E. coli* on each culture plate. From left to right: Mueller-Hinton, MacConkey, and whole blood agar plates. C: Separate experiment in an ascitic rat with cirrhosis that was administered a high amounts of Green Fluorescent Protein-marked *E. colis* harvested after 6 hours. Fluorescent *E. colis* can be seen not only in high density along the GI tract but also in cultures obtained from MLNs as well as in ascites. This experiment was performed in cooperation with Rainer Wiest from the University Hospital Regensburg (Teltschik et al., 2012).

3.4.1.3 Increased bacterial translocation in LC can not be explained by intestinal inflammation

Intestinal tissue from cirrhotic rats with and without BT and healthy controls were scored to investigate whether the expression changes of antimicrobial and related peptides might be due to a secondary effect of inflammation. As shown before (Saitoh et al. 1999), liver cirrhosis was associated with intestinal inflammation (figure 22) but without striking differences between the cirrhosis groups with and without BT. No significant differences between both cirrhotic groups were observed throughout the different tissues in the ileum, coecum and colon (figure 22).

score	0	1	2	3	4
inflammatory infiltrate	none	focal	circumferential mild	circumferential moderate	circumferential severe
mucosal architecture	intact	intact	focally damaged	damaged	loss

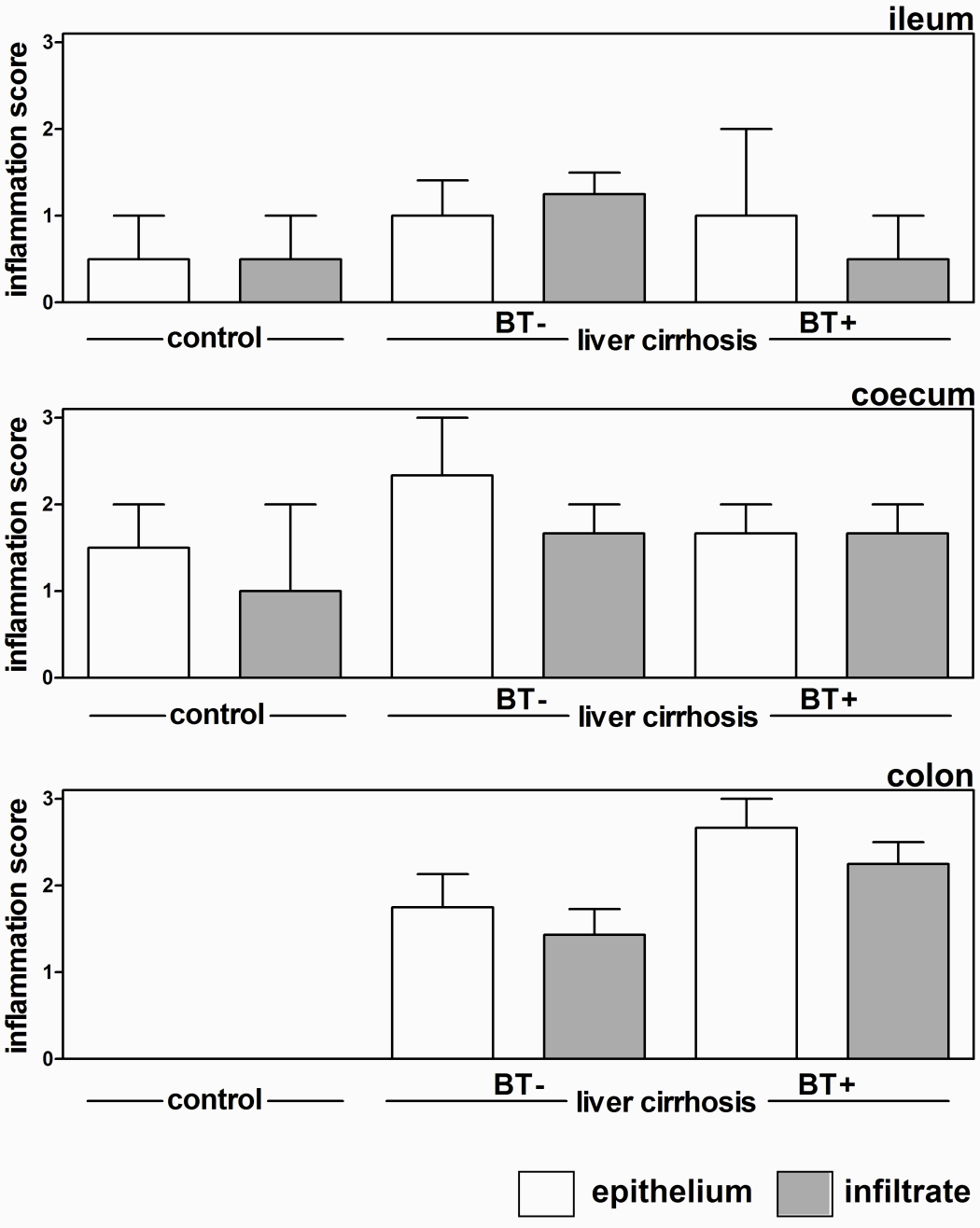

Figure 22: Inflammation score in rats with liver cirrhosis without (LC-) and with bacterial translocation (LC+). Cross sections of the ileum, coecum and colon were haematoxilyn-eosin stained and the histological damage was scored. The degree of inflammatory infiltrate and the mucosal architecture were graded from 0 to 4. No significant differences between the groups were observed (Teltschik et al., 2012).

3.4.1.4 Expression of some Paneth cell products in LC rats is decreased

Expression levels of different antimicrobial peptides and related products were analysed with quantitative real-time PCR in different parts of the intestine from rats with LC and BT, rats with LC without BT and healthy controls. Reduced expression of Paneth cell defensins was found to be associated with BT throughout the whole intestinal tract (figure 23). Especially cryptdin 5 and 7 expression was significantly decreased in rats suffering from LC and BT. These changes were most pronounced in the proximal (crypt 5 $p = 0.02$; crypt 7 $p=0.008$) and distal ileum (crypt 5 $p=0.02$; crypt7 $p=0.01$). Expression of these two defensins was almost undetectable in the coecum and the colon from LC rats with BT in comparison to LC rats without BT; these latter exhibit even increased levels of cryptdin 5 and 7 in comparison to the healthy control group, whereas lysozyme was significantly upregulated in the BT group in comparison to controls. The hepatocarcinoma–intestine–pancreas/pancreatic–associated protein 3 (HIP/PAP3), a member of the Reg family of AMPs expressed in Paneth cells, showed a diverse picture with also almost undetectable expression in the coecum and colon of LC+BT rats.

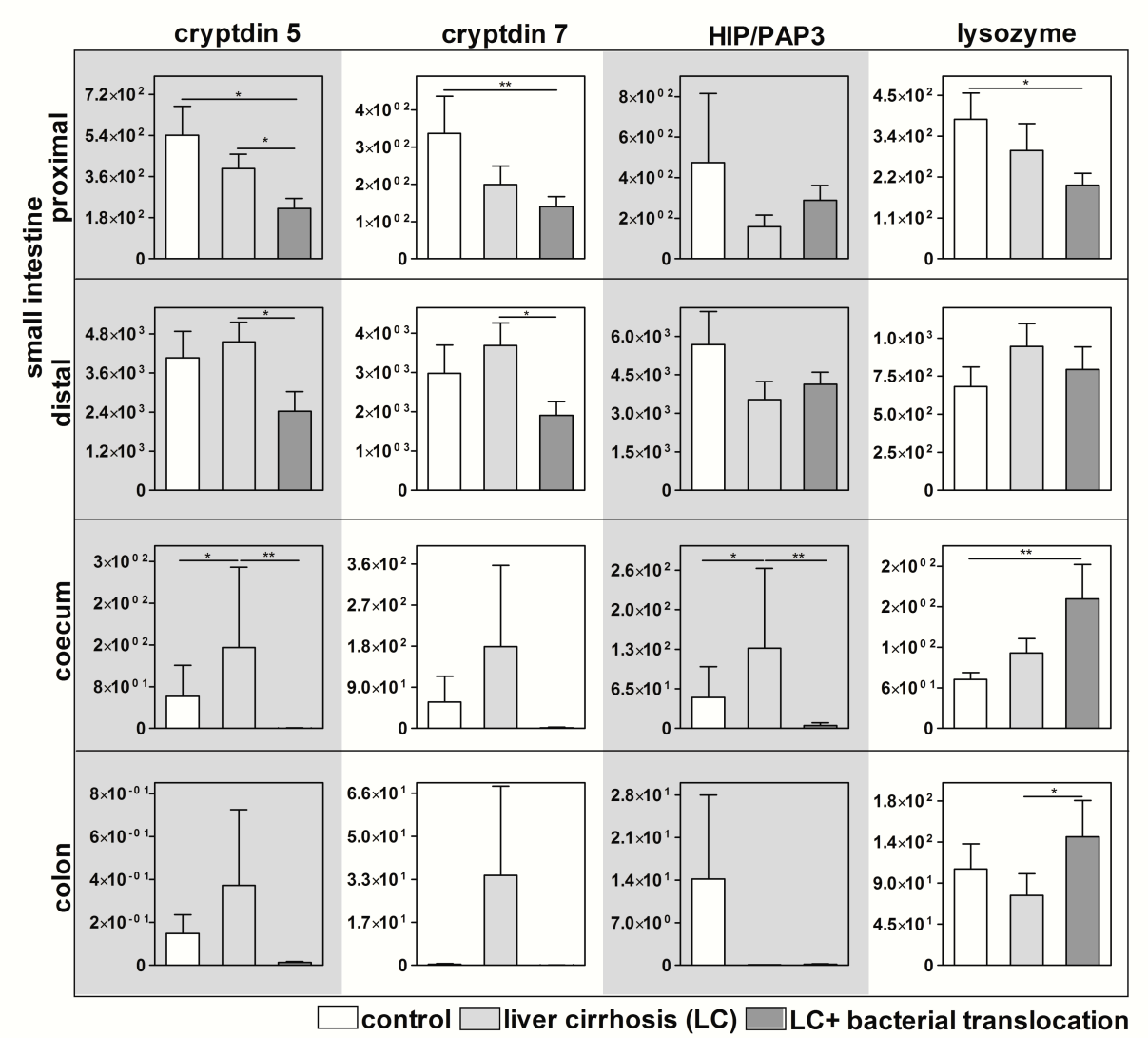

Figure 23: Expression of Paneth cell antimicrobial peptides in liver cirrhosis without (LC-BT) and with bacterial translocation (LC+BT). Expression of cryptdin 5, cryptdin 7, hepatocarcinoma-intestine-pancreas/ pancreatic-associated protein 3 (HIP/PAP3) and lysozyme in proximal and distal small intestine, coecum and colon of controls, LC-BT group and LC+BT group (n=15) was assessed by real-time qPCR. Means (+/- SEM) of transcript copies are shown per 1000 copies β-actin, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Teltschik et al., 2012).

3.4.1.5 Expression of different non-Paneth cell products in rats with liver cirrhosis

Measuring the expression levels of the non-Paneth cell antimicrobials β-defensin 1 (BD1), β-defensin 2 (BD2), CRAMP, which is the rat analogue to cathelicidin and Neutrophil protein 3 (NP3), revealed much lower overall expression, similar to what was observed for the Paneth cell products (figure 24). BD1, which is produced by normal enterocytes, was upregulated in the proximal and distal small intestine and in the coecum of rats with LC+BT. The observed up-regulation was

most pronounced in the proximal ileum (p=0.006). The CRAMP expression in the coecum of rats with liver cirrhosis is significantly decreased (p=0.0027), this effect is even stronger in the subgroup with BT (p=0.0002).

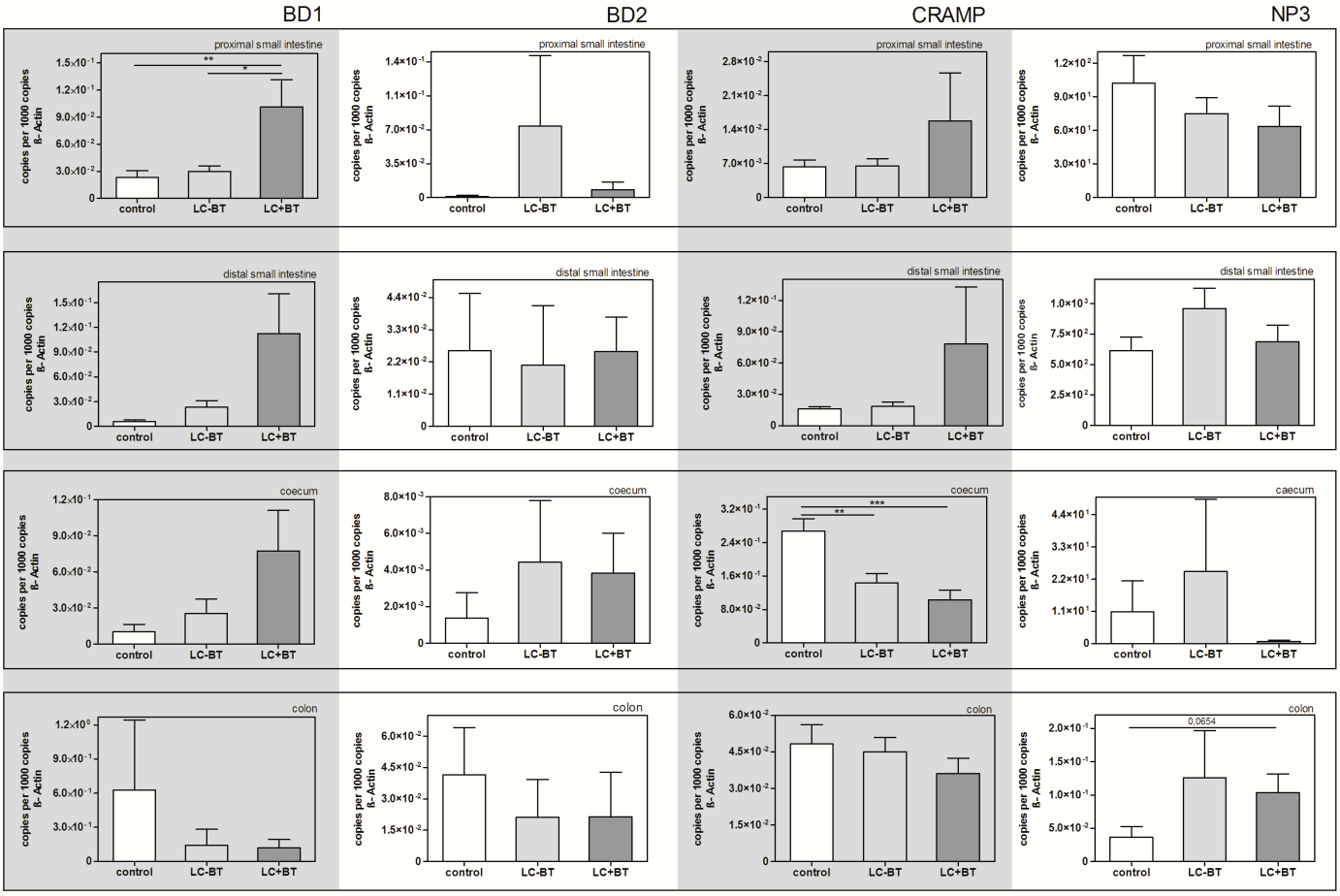

Figure 24: Expression of non-Paneth cell antimicrobial peptides in liver cirrhosis without (LC-BT) and with bacterial translocation (LC+BT). Expression of β-defensin 1 (BD1), β-defensin 2 (BD2), the rat analogue to cathelicidin (CRAMP) and the neutrophil protein (NP3) in the proximal and distal small intestine, coecum, and colon of the controls, LC-BT group, and LC+BT group (n=15) was assessed by real-time qPCR. Means (6SEM) of transcript copies are shown per 1000 copies β-actin, *p<0.05, **p<0.01, ***p<0.001 (adapted from Teltschik et al., 2012).

3.4.1.6 Antimicrobial activity in rats with liver cirrhosis and controls shows significant differences

As already published as a part of my diploma thesis: "*Gestörte Defensinabwehr als mögliche Ursache einer bakteriellen Translokation bei Leberzirrhose*" at the University of Hohenheim in 2009, the mucosal antimicrobial activity (% of killed bacteria) in rats with liver cirrhosis with bacterial translocation (LC+BT) against different commensal bacteria is reduced in the distal small intestine. In the normally sterile proximal intestine, the functional antimicrobial activity in rats with cirrhosis against *Enterococcus faecalis* ATCC 29212 and *Bacteroides fragilis* ATCC 25285 was

comparable to that of controls but approximately doubled against *E. coli* K12 and *Bifidobacterium adolescentis* Ni3, 29c, with no difference between BT and non-BT (figure 25). However, in the distal ileum diminished activity against *E. coli* and *Enterococcus faecalis* was found in the rats with cirrhosis with BT compared with non-BT. A similar effect was detected in the coecum against *Bacteroides fragilis* and in the colon against *Bifidobacterium adolescentis*.

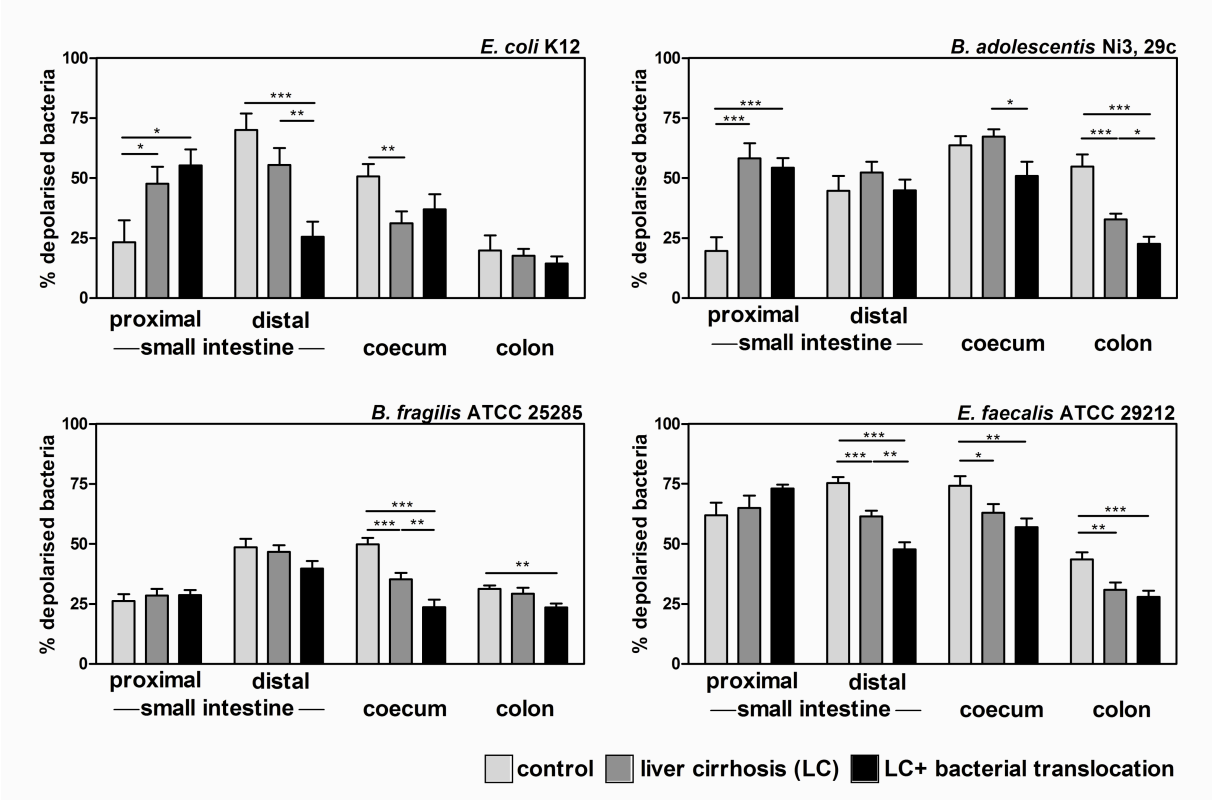

Figure 25: Antimicrobial activity (% of killed bacteria) in rats with liver cirrhosis without (LC-BT) and with bacterial translocation (LC+BT)*. Data are based on protein extracts from controls, LC-BT group and LC+BT group (n=15) against *E. coli K12, B. adolescentis* Ni3, 29c, *B. fragilis* ATCC 25285, *E. faecalis* ATCC 29212. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ (Teltschik et al., 2012).

* these data are already published as part of my diploma thesis: "Gestörte Defensinabwehr als mögliche Ursache einer bakteriellen Translokation bei Leberzirrhose" at the University of Hohenheim in 2009.

3.4.2 Portal vein ligation rat model

The portal vein ligation (PVL) model was chosen in order to examine whether the changes in antimicrobial peptide expression could be related to the phenomenon of portal hypertension per se. This model is known to lack hepatic parenchymal cell damage as well as Kupffer cell dysfunction. Importantly, this PVL animal model is characterized by a high rate of bacterial translocation to mesenteric lymph nodes (Neugebauer et al. 2008). Portal hypertension was induced surgically in aseptic conditions as it has been described before (Lopez-Talavera et al. 1996).

3.4.2.1 mRNA expression of 4 antimicrobial peptides in PVL rats seems to be unchanged

Measuring the expression levels of cryptdin 5, cryptdin 7, HIP/PAP3 and lysozyme in the proximal and distal small intestine, in the coecum and the colon of rats with portal vein ligation and in healthy control rats did not reveal significant differences in these two groups for all four measured products (figure 26).

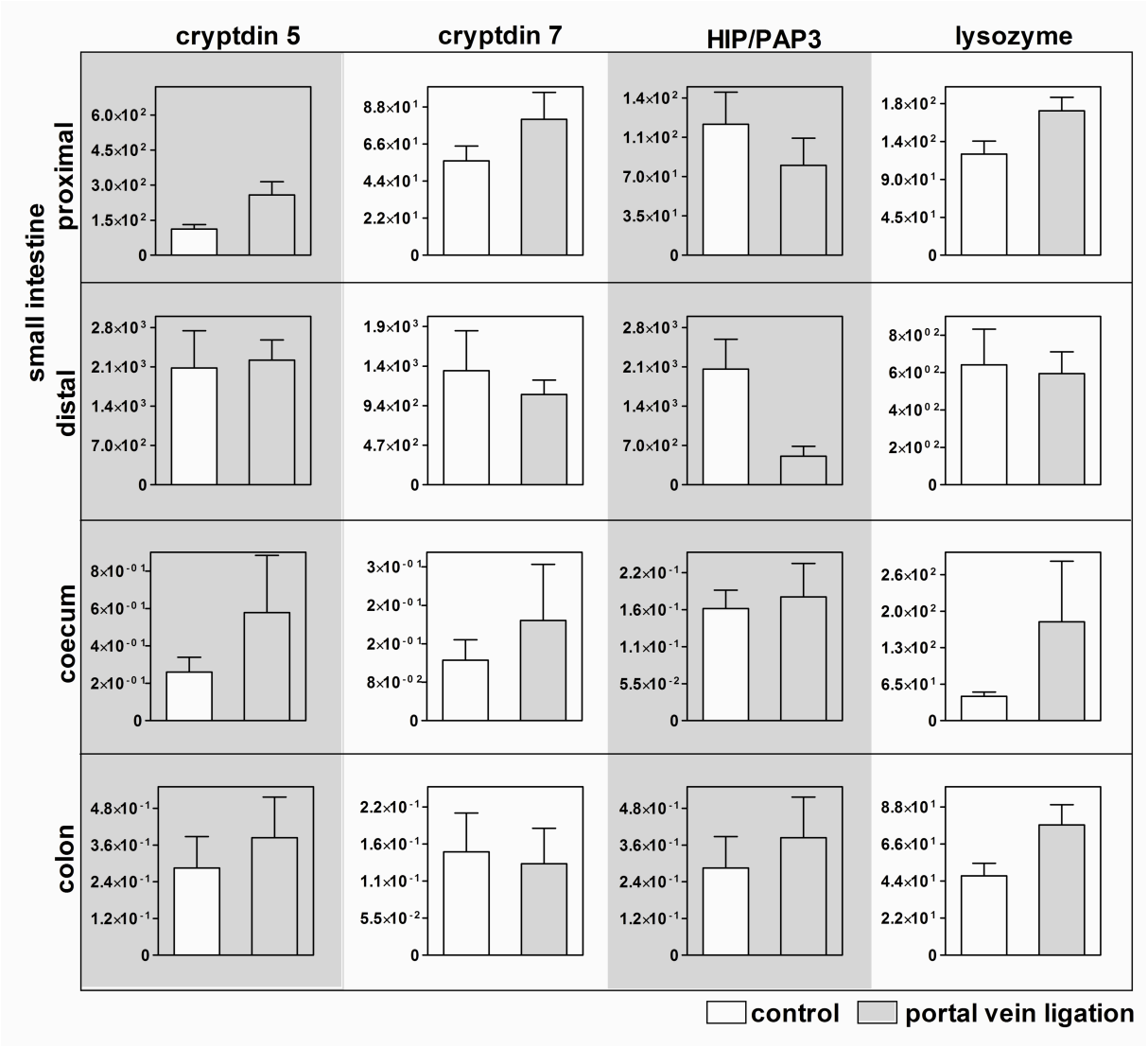

Figure 26: Expression of Paneth cell antimicrobial peptides in rats with portal vein ligation.
Expression of cryptdin 5, cryptdin 7, hepatocarcinoma-intestine-pancreas/ pancreatic-associated protein 3 (HIP/PAP3) and lysozyme in proximal and distal small intestine, coecum and colon of controls (n=6) and rats with portal hypertension (PVL) (n=6) was assessed by real-time qPCR. Means (+/- SEM) of transcript copies are shown per 1000 copies β-actin (Teltschik et al., 2012).

3.4.2.2 Antimicrobial activity in PVL rat intestine and controls seems to be unchanged

Measuring the antimicrobial activity against the same strains used for measuring the antimicrobial activity in rats with liver cirrhosis revealed slightly up-regulated activity against *E. coli* K12 in the proximal and distal ileum and the coecum and against *Bifidobacterium adolescentis* Ni3, 29c in all parts of the intestine. No differences between controls and PVL rats were observed against *Enterococcus faecalis* ATCC 29212 and *Bacteroides fragilis* ATCC 25285 (figure 27).

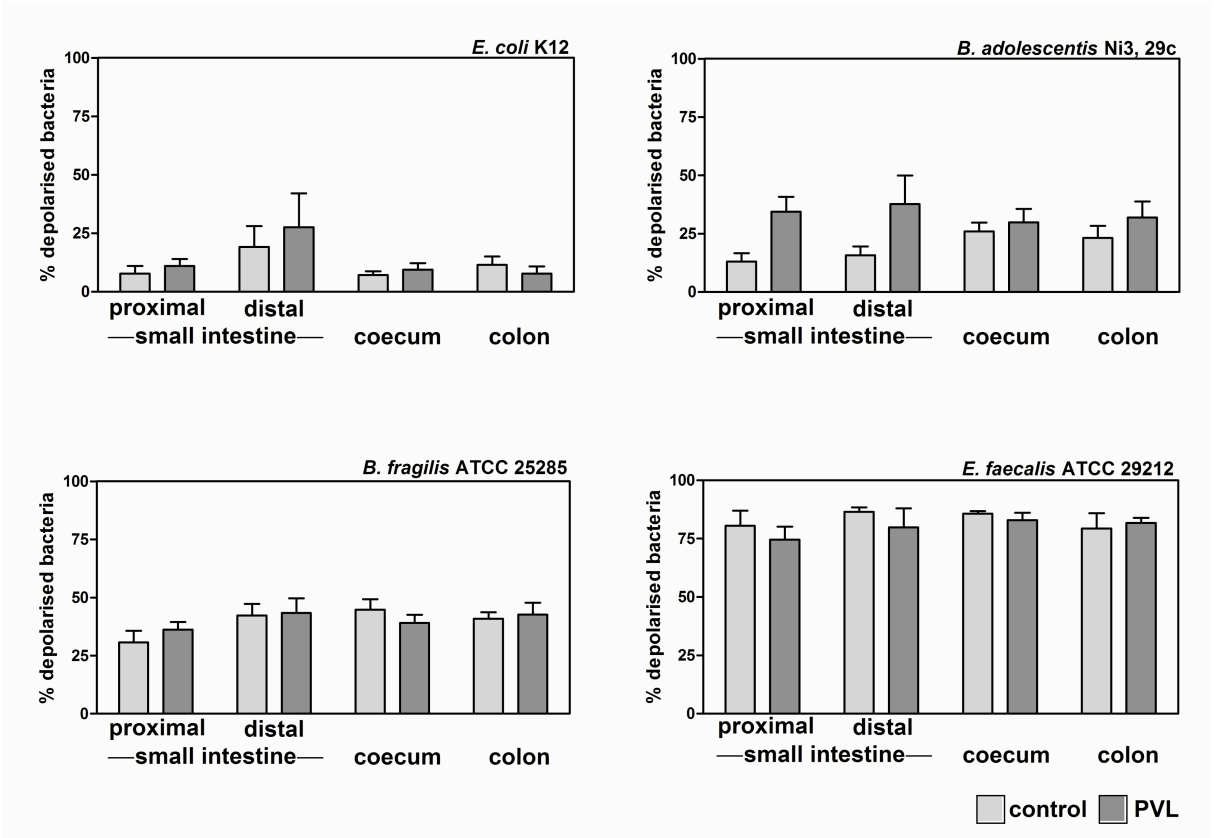

Figure 27: Antimicrobial activity (% of killed bacteria) in rats with portal hypertension. Data are based on protein extracts from controls (n=6) and rats with portal hypertension (PVL) (n=6) against *E. coli* K12. *B. adolescentis* Ni3, 29c, *B. fragilis* ATCC 25285, *E. faecalis* ATCC 29212 (Teltschik et al, unpublished).

These findings emphasize the specificity of the findings in rats with liver cirrhosis. Also, these data suggest that BT in acute vein ligation is caused by a different mechanism, which seems to be independent of the expression levels of some major AMPs.

4. DISCUSSION

In the recent years, the balance between our gut immune system and our gut microbiota has gained popularity in research regarding multiple diseases but also as a crucial part of human health in general. Today many questions remain unanswered when considering all the mechanisms involved in the maintenance of such a beneficial homeostasis. The aim of this study was to shed light on some of these critical homeostasis-promoting functions. A particular emphasis was placed on the host side, but the microbial side was also considered and studied in functional models. One disorder at the forefront of disturbed host microbe interaction is Crohn´s disease, which also involves defects in the Wnt signaling pathway (Beisner et al. 2014; Koslowski et al. 2012; Wehkamp et al. 2007). Other factors of this important pathway were studied to generate new findings, which help to explain what enables commensal microbiota to enter the mucosa and cause inflammations. As described previously, AMPs, which are in part regulated by the Wnt signaling pathway influence the intestinal microbiota (Salzman et al. 2003, 2010), and a disbalance in this system can disturb the homeostasis thereby becoming a predisposing factor in the pathogenesis of CD (Ostaff, Stange, and Wehkamp 2013). It was able to show that LRP6, an important Wnt co-receptor directly regulates Paneth cell HD-5 and HD-6 expression levels (Koslowski et al. 2012b), while LRP5 on the other hand is neither changed on the transcriptional expression level in patients, nor functionally involved in the regulation of these important α-defensins *in vitro*. Finally, this second Wnt co-receptor, while displaying a great heterogeneity in multiple studied populations, showed no genetic association with any subgroup of CD. TCF-1 on the other hand, which is also directly linked to HD-5 and HD-6 expression, displays reduced mRNA in ileal CD patients. A knockout of this important Wnt transcription factor is furthermore linked to lower levels of mouse Paneth cell cryptdins *in vivo* (Beisner et al. 2014).

Studying antimicrobial function in humans is limited more or less to descriptive data. Alternatives are animal models. For analysing the function and role of antimicrobial peptides a liver cirrhosis rat model was chosen. In severe liver cirrhosis, another disastrous gastrointestinal disorder, bacterial translocation through the gastrointestinal mucosa to mesenteric lymph nodes is common and a pathogenesis-promoting event (Wiest, Lawson, and Geuking 2014). This could be due to impairments in barrier function, and therefore due to changes in antimicrobial

defense. Mucosal adherent bacteria are seen in IBD, which has been linked to defective expression of antimicrobial peptides (Perminow et al. 2010). It was tried to elucidate if and how antimicrobial defense at the intestinal barrier plays into a gut liver axis under regard of bacterial translocation and the onset or maintenance of cirrhosis. In this context, it was possible to show that indeed, differences in the expression of Paneth cell defensins and other AMPs are linked to bacterial translocation events (Teltschik et al. 2012). These results, as well as the above mentioned data on Wnt signaling factors in ileal CD will be discussed in further detail in the following chapters.

4.1 Low-density lipoprotein receptor-related protein 6 (LRP6)

The analysis on the functional role of Wnt in ileal CD´s pathogenesis was started with studies on LRP6. To confirm that this important Wnt co-receptor is expressed at the bottom of crypts, where the Paneth cells are located, immunohistochemical staining of human small intestinal tissue slices was performed. LRP6 could be detected in the whole epithelium suggesting an expression also at the bottom of the crypt where active Wnt signaling is important for the α-defensin expression. To get an idea whether this expression can translate into a functional involvement, *in vitro* experiments using a HEK-293 cell line were performed. Unfortunately, no small intestinal cell line exists, and colonic cell lines are unsuitable for studying Wnt related mechanisms, since the exclusively descend from cancer tissue, which harbors mutations that constitutively increase Wnt signaling activity (Teltschik et al. 2012). HEK-293 cells on the other hand originate from human embryonic kidney cells and harbor no Wnt affecting mutations. It is therefore often the cell line of choice when studying this pathway. The problem using this cell line is that tissue-specific properties and interactions are ignored.

LRP6 overexpression in HEK-293 cells showed promoter transactivation of the target genes HD-5 and HD-6, confirming a direct role of LRP6 in regulating both these Paneth cell α-defensins. Transfection with a TopFlash luciferase reporter construct, which is an artificial promoter that is very responsive to Wnt signaling because it contains multiple repeated copies of wild-type TCF/LEF-binding sites (Korinek et al. 1997; van de Wetering et al. 1997), showed corresponding effects. This β-catenin dependent induction of Wnt signaling by LRP6 overexpression is consistent with former findings (MacDonald et al. 2011). These results show the importance of

LRP6 in mediating β- catenin dependant Wnt signaling and highlights it's importance in regulating α- defensin expression.

4.2 Low-density lipoprotein receptor-related protein 5 (LRP5)

Because LRP6 holds a critical role in mediating regulation of α-defensins (M. J. Koslowski et al. 2012) it was hypothesized that the LRP6 homologue LRP5 is also involved in this regulation. Measuring mRNA levels in the Stuttgart cohort and a pediatric cohort from Norway revealed no significant differences between CD patients (in ileal and colonic subgroups) and a healthy control group. This leads to the conclusion that the observed decrease in human α-defensin 5 in patients with ileal CD (L1+L3) is not additionally due to changed expression levels in LRP5. If the receptor is however functionally involved in their regulation still remained to be answered. To further elucidate the functional role of LRP5, overexpression experiments with HD-5 or either HD-6 co-transfection in HEK-293 were performed. After overexpression of LRP5 and subsequent co-transfection with either a HD-5 or HD-6 promoter construct, Wnt signaling was stimulated with either Wnt1 or Wnt3a. To exclude effects due to LRP6 activity, LRP6 was knocked-down with siRNA. No significant induction of the HD-5 and HD-6 promoter activity was observed, also after stimulation with Wnt3a which was already described to increase LRP5 signaling activity before (Grumolato et al. 2010), indicating that LRP5 is not required to induce α-defensin expression. These results are consistent with data from MacDonald et al. (MacDonald et al. 2011). The group could show that the signaling capacity of LRP6 is much stronger than the LRP5 signaling capacity. But again, since LRP5 could have other important functions in maintaining epithelial integrity, it is not possible to exclude that it might nonetheless be involved in CD pathogenesis. The failed induction by Wnt3a and Wnt1 could also be due the used method. Although Wnt3a and Wnt1 are described to activate canonical Wnt pathway, many functions of the different Wnt ligands and which pathways they activate are still unknown (Dijksterhuis, Petersen, and Schulte 2014). The used artificial Wnt stimulants were not able to lead to a stable stimulation and it was not possible to observe stimulating effects at all. This could also be due to the fact that it seems very difficult to obtain pure biological active Wnts (Dijksterhuis, Petersen, and Schulte 2014). Cells stimulated with Wnt3a conditioned medium showed effects on Wnt signaling in HEK cells (Surmann-Schmitt et al. 2009).

A coding allele, the AIle1062Val SNP (rs2302685) in the LRP6 gene is associated with the development of early onset of small intestinal CD, arguing for the analysis of mutations in the LRP5 gene to elucidate whether CD patients, or a subgroup of CD patients, carry single nucleotide polymorphisms (SNPs) which could be predisposing in the development of CD. To investigate the potential role of LRP5 in the pathogenesis of CD analysis of 45 SNPs (for detailed information, see figure 14) in the gene was performed. These SNPs, made up of exonic, coding (non-synonymous) SNPs, exonic synonymous SNPs but also intronic SNPs, were analysed in three different European cohorts (Stuttgart, Vienna, Leuven). After sub-grouping the samples according to gender, age, age of onset of disease, behaviour of disease and location (L1, L2 and L3 and additional involvement of the upper GI-tract (L4)), the data set was analysed. Although significant differences in single cohorts were observed, overall analysis relativized the observed differences. This could be due to the fact the LRP5 gene seems to be very heterogeneous in the studied populations. An at least partial explanation for this observation could also be the essential role that the protein plays in skeletal homeostasis and some of the bone density related variability and diseases within populations seem to be for example linked to SNPs in LRP5 (Yi et al. 2013). Furthermore, familial exudative vitreoretinopathy has also been linked to mutations in this gene (Toomes et al. 2004).

That SNPs associated with bone mineral density (BMD) variability, might not be under high selection pressure could be due to the fact that some of these SNPs could also have protecting abilities in other contexts. This is a possible explanation why the LRP5 gene seems to be so heterogeneously in regards to its SNP allele frequencies. The broad range of SNPs which are associated with different diseases or phenotype variability also make it hard to unmask potential effects in the IBD cohort since it is not possible to stratify the samples according to such possible confounders. Around 15 SNPs in the LRP5 gene are associated with the development of familial exudative vitreoretinopathy, most of them influence the amount of produced LRP5 (Qin et al. 2005; Toomes et al. 2004; Qin et al. 2008). Around 5 SNPs in the LRP5 gene are furthermore associated with juvenile primary osteoporosis. This leads to reduced signaling capacity of LRP5 which disrupts the regulation of BMD and causes early onset osteoporosis (Hartikka et al. 2005; Korvala et al. 2012). Osteoporosis-pseudoglioma syndrome, which is associated with extremely low bone mineral density, is associated with more than 40 SNPs in the LRP5 gene. Patients suffering from this disease have multiple bone fractures and often form eye abnormalities. A

lot of the associated mutations obviate any production of LRP5 protein, others cause abnormal proteins not able to insert into their proper position and thereby disables them to perform their proper function (Ai et al. 2005; Gong et al. 2001; Levasseur, Lacombe and de Vernejoul 2005). Low BMD and osteoporosis is partly believed to be developed secondary due to medication and malabsorption caused by inflammation (Reinshagen 2008). But medication and inflammation can only explain parts of the observed effects and genetic predispositions predominantly in the Wnt pathway confer major risks (Ali et al. 2009). But not only low BMD is observed in patients with SNPs in the LRP5 gene, some SNPs are associated with increased bone mineral density. Persons with increased bone mineral density sometimes suffer from abnormal bone growth and skeletal architecture but can also be completely free of symptoms (Van Wesenbeeck et al. 2003; Boyden et al. 2002). Again, these examples suggest a high variability due to many SNPs in the gene, which can influence highly variable phenotypes within a population. In the cohort it was not possible to stratify since the knowledge in the case history of the patients regarding other diseases, as well as their BMD was not available. Another, methodical, reason for the heterogeneous results in the three cohorts could be that the used cohorts are too small. Further experiments should be conducted in bigger cohorts.

4.3 T cell transcription factor 1 (TCF-1, TCF7)

Since it could be shown that the Wnt transcription factor TCF-4 shows aberrations in ileal CD patients and is furthermore important for the regulation of HD-5 and HD-6 (Wehkamp et al. 2007), it was decided to study TCF-1 in this context. Immunohistochemistry revealed expression of active TCF-1 in Paneth cells and also in Paneth cell progenitors above Paneth cells in the crypt. That active Wnt signaling is essential for the regulation of α-defensins in Paneth cells, as mentioned, is in line with former findings from our group (Wehkamp et al. 2007) and also with findings from Gregorieff et al., who could show that TCF-1 is also found to be expressed in epithelial cells in the crypt base (Gregorieff et al. 2005). In this context it makes complete sense that active TCF-1 is detected in α-defensin producing Paneth cells. Measuring the expression of TCF-1 and the active isoform 1 in the Stuttgart cohort showed significantly reduced levels of both in small intestinal biopsies from patients with ileal CD and also in patients with ileocolonic disease. Expression of TCF-1 and active TCF-1 isoform 1 were furthermore analysed in a second cohort from Norway comprising children with CD and a healthy control group. As in the Stuttgart cohort,

pediatric patients with ileal and ileocolonic disease showed significantly reduced expression levels of TCF-1 and active TCF-1 isoform 1 (Beisner et al. 2014). These findings further strengthen the finding that the Wnt signaling pathway has an important role in the pathogenesis of CD. To further study the role of TCF-1 *in vivo*, analysis of the mRNA levels of different cryptdins (mouse α-defensins) in a TCF-1 knockout mouse model was performed. Analysing these cryptdins and also other Paneth cell products showed that, when TCF-1 is depleted, the levels of some cryptdins are slightly decreased (figure 19). These trends are weaker, but in line with former findings from our group, which showed that in TCF-4 heterozygous knockout mice, the levels of cryptdins are decreased (Wehkamp et al. 2007, 4). Paneth cell numbers are unchanged in TCF-1/TCF-3 double knockout mice (J. H. van Es et al. 2012), indicating that the observed effects are not due to lower Paneth cell numbers in the TCF-1 knockout mice. Possible is that stronger effects due to TCF-1 depletion could be compensated by TCF-4, which has overlapping functions, resulting in lower effects. Another possibility is that in mice, other mechanisms or regulatory influences are important in these parts of the intestine. Neonatal TCF-4 knockout mice display a complete lack of intestinal crypts, which is also seen in conditional knockout of β-catenin, resulting in completely blocked cell proliferation (van Es et al. 2012). Analysing the expression of total cryptdins with a pancryptdin assay in the duodenum, jejunum, and ileum of the same mice showed decreased cryptdin levels in the jejunum but not in the duodenum and the ileum. Interestingly, Reg3γ and lysozyme as two other Paneth cell products, not belonging to the group of cryptdins showed a different trend; lysozyme mRNA expression is similar to the observed trends in cryptdin expression whereas Reg3γ displays even higher expression in homozygous TCF-1 knockout mice (figure 20), prompting for an other regulation mechanism than cryptdins. In addition Reg3γ is also produced by enterocytes and lysozyme by macrophages and certain cells in the lamina propria (Bevins and Salzman 2011; Cash et al. 2006). This inconclusive data suggests slight effects on cryptdin expression in at least the jejunum of homozygous TCF-1 knockout mice. As mentioned before, Paneth cell number is likely unchanged in these mice suggesting another reason than Paneth cell number to be responsible for the observed effects.

4.4 Examination of the functional relevance of antimicrobial peptides in the intestine in two different animal models

Animal models are a possibility to study aspects or answer questions when the options in humans are limited due to limited sample availability and ethical issues. Herein two different rat models were used to better understand the role of defensins and other antimicrobial peptides *in vivo*. Severe liver cirrhosis is linked to impairments in barrier function, which leads to bacterial translocation (BT) over the mucosa and to the mesenteric lymph nodes (Wiest, Lawson, and Geuking 2014). This situation is similar to the situation in inflammatory bowel diseases. In both, commensals of the gut play a central role in the pathogenesis of the disease. It is likely that a connection between the liver and the gut together with other unknown hepatic factors and antimicrobial peptides at the intestinal barrier could have impact on the mucosal immune system. The LC rat model comprised rats with a CCl_4 induced liver cirrhosis. The second model was used to exclude the possibility that the observed changes in the LC model are only due to liver cirrhosis- induced portal hypertension. The portal vein ligation (PVL) model comprised rats with a portal vein ligation, imitating the congestion of blood, which is typically observed in liver cirrhosis due to destroyed liver tissue, but without the cirrhosis. Measuring the mRNA levels of different antimicrobial and related peptides revealed significantly decreased levels of cryptdin 5 and 7 in rats with liver cirrhosis and BT in comparison to rats with liver cirrhosis without BT and to healthy control rats. In PVL rats, no significant decreased levels were observed in comparison to controls, arguing for a mechanism, which seems to be independent of the portal hypertension as it was observed in both models. The mechanisms causing the BT in the PVL rats is indeed likely different, because in the PVL group, all animals develop BT short after vein ligation although the defensin expression is not significantly changed, whereas BT in the CCL_4 model was clearly associated with reduced AMP levels along with decreased cryptdin mRNA levels in the LC+BT group, the antimicrobial activity of protein extracted from intestinal tissue is also decreased against different bacterial strains. Because inflammation is associated with liver cirrhosis, the inflammation state of the rats was additionally assessed and it could be shown that effects were not only a consequence of intestinal inflammation as the inflammation score did not correlate with the decrease in AMPs (Teltschik et al. 2012). The mechanisms, which could be critical for the observed decrease in Paneth cell cryptdins in the subgroup with liver cirrhosis and BT remain unclear but the decreased antimicrobial activity in these

animals are likely not mediated by a changed β-defensin (BD) expression. As mentioned, the picture is more diverse as for BD1, even a slight up-regulation in the LC+BT group was observed. The working group around Salzman et al. could show that Paneth cell defensins have the potential to inhibit BT in a transgenic mouse model (Salzman et al. 2003). Those findings support the data by indicating that the decrease in α-defensins or cryptdins could favour BT.

That BT is only linked to a subgroup of rats with liver cirrhosis could possibly be explained by genetic predispositions. Especially because it is known that mutations in the NOD2 receptor, which has been linked to Paneth cell defensin expression (Bevins, Stange and Wehkamp 2009), and other genetic mechanisms are linked to bacterial peritonitis and even death in liver cirrhosis patients (Appenrodt et al. 2010; Bruns et al. 2012). As mentioned above, variants in this gene are linked to ileal Crohn´s disease and thereby to decreased antimicrobial peptide expression in the Paneth cells leading to a shift in the bacterial composition which could promote bacterial overgrowth and translocation (Wehkamp et al. 2004; Wehkamp et al. 2005). In healthy individuals, the complex interplay between microbes and intestinal mucosal defence leads to a well-balanced cooperation of commensals and host defence against invading pathogens which also prevents inflammation (Hooper and Gordon 2001). Assuming that in predisposed animals liver cirrhosis favours the disruption of this balance because of a misbalanced production of α-defensins especially in the small intestine, these changes may lead to a disturbed microbial composition promoting BT (Morencos et al. 1996; Gupta et al. 2010; Natarajan et al. 2006). To better understand the underling mechanisms it would be necessary to perform genetic analysis in LC rats.

4.5 Conclusion

Taken together, within this work it was possible to shed light on multiple mechanisms involved in balancing host microbe interactions at the intestinal barrier. It was able to further highlight the Wnt pathway as an important contributor to ileal CD. Amongst other mechanisms, Wnt signaling could be identified as a relevant factor in the transcriptional expression of the Paneth cell antimicrobial peptides HD-5 and HD-6. Both these defensins are known to be crucial in maintaining a beneficial homeostasis towards microbiota at the small intestinal barrier. Furthermore, it could be demonstrated that bacterial translocation in a rat model of liver cirrhosis can be linked to lower levels of these defense mediators, but also other AMPs in the gut of

affected animals. While many of the underlying mechanisms in the complex and multifactorial inflammatory bowel disease Crohn's disease are yet to be elucidated, identifying Wnt and it's role in Paneth cell defects in patients, surely provides new possibilities to potentially one day develop curative therapy.

The diminished HD-5 and -6 levels could provide a possible target for therapy; the administration of these two components could potentially restore the low levels. For a proper function, Wnt signaling must be tightly regulated. Interventions in this pathway could lead to an increased risk of developing cancer, because this pathway regulates cell growth and proliferation. Genesis of gastric cancer is in more than 70% associated with a gain in Wnt signalling (Ooi et al. 2009). Kaler et al. could show, that colorectal cancer is coupled with a gain of wnt signaling (Kaler et al. 2009). Therefore it requires more knowledge to use this promising working point. A better understanding of the homeostasis between bacteria and the human gut could as well provide new treatment targets. Further research is needed to gain more knowledge about the microbiota-gut-axis.

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7. APPENDIX

Primer used in Sequenom SNP genotyping assay: all Primers had a purification scale level of 0.04 µmol and were desalted. Primers were designed using SNPdb sequence information or Ensemble and subsequently Sequenom software (see methods part). Primers used in Mulitplex-PCRs for the amplification of sequences carrying the variation have the additional specification 1st-PCRP or 2nd-PCRP. PEX reaction primers were additionally named UEP_SEQ.

1	rs2137328	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTCATCACGTTCTGGCAGTAG ACGTTGGATGCTAGGATTACAGGCATGAGC GGTTGTGATAGAAGTTATTGTTTTG
2	rs11606508	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTCAAAGGCTTGTTTTCAGCG ACGTTGGATGCAGAGTCCAGACATTTGTGC AAGCAACTGGATTTTTCTTAACC
3	rs682429	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGAGGAGAAGAATCTCACCCAG ACGTTGGATGAGACTGGGAGTTCCAACTTG cgaggacGGGCACAGAAGGGGCTC
4	rs4988331	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTTTCTCTCAGTGGCAAGAGC ACGTTGGATGAGAGACAGAGAACAGTGTCC tagaCTGATGGGCCAGAGGTTC
5	rs4988300	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCAAAGGGTGGAACCTGATAG ACGTTGGATGCCCACCTCATTGTCCTTTTC gaAAAAGCATAAACGTGCAAG
6	rs634008	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCCTCTTCGTTCCATTTCCTC ACGTTGGATGTCCCAGGACCTCAGCACAAT aGTTCCATTTCCTCACAGTCT
7	rs312778	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGAACCACACACCCAGCATAAC ACGTTGGATGCCTAATGAGGCCACCTTTAC ccccAGCACCAGCGGAGAC
8	rs314756	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGAGGAGATGGTCACAGCTAAG ACGTTGGATGGCCTTTGACCCTGAGTTTTC ATCTCATCCCCTGCC
9	rs312781	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCCGTGCTGTCATCATCTTAG ACGTTGGATGATTTGTGGCCTATGCCTTCC cccgaTGTCATCATCTTAGGTCTAC
10	rs78219242	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTTCCAGTTTTCCAAGGGAGC ACGTTGGATGCCCCGTCTGGTTCAGGTAG gaAGCGAGGAGGCCATCA
11	rs41494349	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGGAGATGACCACGTTCTGCAC ACGTTGGATGTGTACTGGACAGACGTGAGC GTCTGGTTCAGGTAGGTC

12	rs643981	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCTTGGGCTCATGCAAATTCG ACGTTGGATGTCACGGGTAAACCCTGCTG TGCAAATTCGAGAGAGA
13	rs312788	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGGCCTCAGTTTTCTCATCTGC ACGTTGGATGCTGTGCATCTAGTGAGAACC tATTCCCACCTTCCCT
14	rs121908673	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCTCTCCGGGGACACTCTGT ACGTTGGATGTCAGGATCTCCTTCCTCTTC GGACACTCTGTACTGGA
15	rs638051	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTTTGGGCAGTGGGCTTAGCG ACGTTGGATGGACATAGCTCTGAGAGCCAC TGCCACTCGGGCCACAC
16	CM053969	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCCATTGCACCTGTCTCCACA ACGTTGGATGATGTCGGTGAAGTCCGGCGT ggcgtACGGACCTACGGAGGATCT
17	rs4988319	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTCCAGGTCAATCTCCCTCTC ACGTTGGATGTCGCACTGCACCCCGTGAT gccatGCCCCAGCCCCCGCC
18	rs4988320	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTGACCCCATGAGTCTGTCTG ACGTTGGATGACATTTAGCCATGTGATGGG TGAGTCTGTCTGGTCTCTG
19	rs491347	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTGAGCCTGCAGAACTGTATG ACGTTGGATGTTTCATCCTGTCCTGAGAGC gggtgAACAGGAAGGTTGAGTTAGG
20	rs686921	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGAAACAAGACGGGTTCAATGC ACGTTGGATGAGCGTCATTTATCCAGCACC TCAATGCCCAAGAGC
21	rs545382	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCAGTCCAGTAGATGAAGTCC ACGTTGGATGATGTTGATGGGACGAAGAGG cccaCCCCAGCAGCGTGAACCC
22	rs2277268	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGGTTGTTATTGGTCTCGAGGG ACGTTGGATGTGAGTGACATGAAGACCTGC TGAAGACCAAGAAGGC
23	rs4988321	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTCTCCCTCGAGACCAATAAC ACGTTGGATGCCAGTAGATGTGGTTGTTGG ggtTCGAGACCAATAACAACGAC
24	rs2306862	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTCCCTCCACACGAGGACTTG ACGTTGGATGATGGGCAAGAACCTCTACTG cccggCCAGCCGCGCCACTTCGATTCT
25	rs4988322	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTGTGGAGGGACTTGGACAAC ACGTTGGATGTACGAGGTGAACACAAGGAC ATCCCACCAAGGGGTAAG
26	rs2242339	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGAACTTGCAGGCCACAGGGTA ACGTTGGATGACATGATCGAGTCGTCCAAC ACGCCCGGCTGCCCC
27	rs61370283	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGTGAGGAACGTCAAAGCCATC ACGTTGGATGTTGGCTCGCTTGATGTTCTG AGCCATCGACTATGACCCA

28	rs2472415	2nd-PCRP	ACGTTGGATGAACTCCTGACCTCGTGATCC
		1st-PCRP	ACGTTGGATGCCTAAGCAGCCTTCTTTTAC
		UEP_SEQ	gggtCCAAAGTGCTAGGATACA
29	rs61889560	2nd-PCRP	ACGTTGGATGACGACCTCAGCATCGACATC
		1st-PCRP	ACGTTGGATGGTGGACGTTGATGGTATTGG
		UEP_SEQ	acccCAGCATCGACATCTACAGCC
30	rs599083	2nd-PCRP	ACGTTGGATGCCCATTGTGGTCGTATCTTG
		1st-PCRP	ACGTTGGATGCAGAACCTCAGAATGTGAGC
		UEP_SEQ	atttTGGTCGTATCTTGATCCTTCA
31	rs554734	2nd-PCRP	ACGTTGGATGACAGGTCACAGCTCTCAATG
		1st-PCRP	ACGTTGGATGTCTTCACCACCGGCCTCATC
		UEP_SEQ	TGCCCAGTGTGTTGTC
32	rs17149104	2nd-PCRP	ACGTTGGATGATGAGGCCGGTGGTGAAGAG
		1st-PCRP	ACGTTGGATGACTTCACCAACATGCAGGAC
		UEP_SEQ	ccccgAGAGGACCTCGCGCTCGGTGCC
33	rs556442	2nd-PCRP	ACGTTGGATGTCTTCACCACCGGCCTCATC
		1st-PCRP	ACGTTGGATGTCTCAATGCGCTTCAGGTCC
		UEP_SEQ	TGTGGCCCTGGTGGT
34	rs11607268	2nd-PCRP	ACGTTGGATGCACGTACAGAACTCCTCCAG
		1st-PCRP	ACGTTGGATGATCGAGCGTGTGGAGAAGAC
		UEP_SEQ	tCAGGCTGACTTCCTCCA
35	rs12416761	2nd-PCRP	ACGTTGGATGAAGCAGAAGTTGAGAGGGAC
		1st-PCRP	ACGTTGGATGAGCGATGGAGGATGTGCGG
		UEP_SEQ	cttcGGCATCCCTGCTGTA
36	rs12417014	2nd-PCRP	ACGTTGGATGGACAGAGGAGAACATCACAC
		1st-PCRP	ACGTTGGATGTGAGTCCCTCTCAACTTCTG
		UEP_SEQ	cccctCAAAACCCAGGCAATGGC
37	rs3736228	2nd-PCRP	ACGTTGGATGTCTTGGCAGAGCCTTGACG
		1st-PCRP	ACGTTGGATGAGACTGTCAGGACCGCTCA
		UEP_SEQ	tCCTCACCGTCACAGTCC
38	rs901824	2nd-PCRP	ACGTTGGATGTAGCACATCCCTGCTCCACT
		1st-PCRP	ACGTTGGATGCCCTTCACAGGTAAGGAGC
		UEP_SEQ	ttTGCCTCCTCCAGATC
39	rs1127291	2nd-PCRP	ACGTTGGATGTCTACTCTTCAAACATTCCG
		1st-PCRP	ACGTTGGATGGAACCCAATGGCCATGGAG
		UEP_SEQ	AACATTCCGGCCACTG
40	rs676318	2nd-PCRP	ACGTTGGATGTCATTTTTCACTGGGCCCTG
		1st-PCRP	ACGTTGGATGACAGAGAGGTCAGATGGAAG
		UEP_SEQ	GGGCTCTTCTGAACC
41	rs3867143	2nd-PCRP	ACGTTGGATGAAGAAAGTCCATGTTGCCAG
		1st-PCRP	ACGTTGGATGCCACATTTCTCGGGAACAAG
		UEP_SEQ	ccttcCCAGTTTGGCTATTTCC
42	rs2472429	2nd-PCRP	ACGTTGGATGGGTAACTTTGCTGTGGAGTC
		1st-PCRP	ACGTTGGATGGATCCTTCAAGGCGTATGTG
		UEP_SEQ	gggtcACTTTGCTGTGGAGTCTCACAT
43	rs12294029	2nd-PCRP	ACGTTGGATGTGATACCCTGGCAAATACGG
		1st-PCRP	ACGTTGGATGCACTTTGCAGATACTGTGTG
		UEP_SEQ	gtGTTGCCATAAACCTTCAAT

44	rs7126340	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGGGGAAACCAATATATTCGTG ACGTTGGATGGGTAAAGGCATACCTTGGAG cTGTGACATTTGCTTTTTTG
45	rs12226585	2nd-PCRP 1st-PCRP UEP_SEQ	ACGTTGGATGCCTCCCACCTTGATTTCTTT ACGTTGGATGCTTACCCCCCACCAAAAAAA aggGATTTTCTGACATTTGACTATG

Overall alle frequencies all cohorts

Leuven

	rs314756	rs676318	rs556442	rs312788	rs1127291	rs638051
	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	SNP 6
	intronic	intronic	exonic V-->V	intronic	exonic A-->V	intronic
LEUVEN MAFs						
Controls	7.87%	5.95%	29.55%	33.94%	0.40%	33.40%
Diverticulitis	23.84%	10.56%	49.40%	46.48%	1.15%	36.88%
IBD	9.26%	8.49%	32.47%	42.66%	1.76%	37.63%
UC	12.50%	5.49%	24.73%	40.22%	1.10%	32.45%
CD	8.18%	9.49%	35.09%	43.49%	1.98%	39.36%
male	10.23%	8.82%	35.38%	45.86%	2.05%	40.52%
female	8.53%	8.81%	31.37%	40.53%	1.64%	35.71%
A1	14.71%	11.76%	35.29%	48.53%	4.41%	33.82%
A2	8.03%	8.99%	31.25%	42.52%	1.37%	39.96%
A3	11.64%	4.79%	30.67%	41.67%	2.05%	33.33%
B1	7.14%	11.43%	36.96%	42.14%	2.14%	35.71%
B2	7.02%	12.07%	31.03%	39.29%	1.72%	35.83%
B3	8.47%	7.69%	34.32%	46.05%	1.67%	43.03%
L4*	7.14%	9.52%	38.10%	50.00%	7.14%	42.86%
ileal CD	8.80%	9.95%	36.41%	46.68%	1.60%	41.70%
male	9.68%	9.68%	39.13%	48.90%	2.13%	44.74%
female	8.13%	10.16%	34.40%	45.00%	1.20%	39.45%
A1	14.00%	18.00%	48.00%	56.00%	4.00%	40.00%
A2	7.88%	8.90%	34.25%	46.83%	1.01%	44.08%
A3	8.75%	10.00%	40.24%	42.31%	2.44%	35.37%
B1	10.23%	14.77%	46.51%	52.27%	2.27%	42.05%
B2	7.55%	12.96%	32.41%	41.35%	0.93%	38.39%
B3	8.80%	7.01%	34.40%	47.12%	1.82%	42.86%
L4*	7.14%	9.52%	38.10%	50.00%	7.14%	42.86%
* the same						

	rs121908673	rs3736228	rs78219242	rs61370283	rs4988320	rs4988319
	SNP 7	SNP 8	SNP 9	SNP 10	SNP 11	SNP 12
	exonic T-->I	exon A-->V	exon L-->R	exon L-->M	intronic	intronic
LEUVEN MAFs						
Controls	0.00%	13.75%	0.00%	0.00%	11.02%	14.46%
Diverticulitis	0.00%	30.59%	0.00%	0.00%	13.07%	15.70%
IBD	0.00%	16.21%	0.00%	0.00%	15.42%	15.62%

UC	0.00%	12.92%	0.00%	0.00%	11.11%	14.67%
CD	0.00%	17.27%	0.00%	0.00%	16.85%	15.93%
male	0.00%	16.37%	0.00%	0.00%	17.37%	15.98%
female	0.00%	15.73%	0.00%	0.00%	14.42%	14.93%
A1	0.00%	10.61%	0.00%	0.00%	16.18%	13.24%
A2	0.00%	15.14%	0.00%	0.00%	16.82%	15.74%
A3	0.00%	19.86%	0.00%	0.00%	10.00%	16.22%
B1	0.00%	15.22%	0.00%	0.00%	17.14%	15.22%
B2	0.00%	13.56%	0.00%	0.00%	18.42%	12.07%
B3	0.00%	18.75%	0.00%	0.00%	16.37%	17.24%
L4*	0.00%	20.00%	0.00%	0.00%	14.29%	23.81%
ileal CD	0.00%	18.04%	0.00%	0.00%	18.08%	17.06%
male	0.00%	18.82%	0.00%	0.00%	19.23%	17.58%
female	0.00%	17.46%	0.00%	0.00%	17.21%	16.67%
A1	0.00%	14.58%	0.00%	0.00%	22.00%	16.00%
A2	0.00%	17.67%	0.00%	0.00%	18.40%	18.06%
A3	0.00%	21.25%	0.00%	0.00%	15.38%	15.00%
B1	0.00%	17.44%	0.00%	0.00%	20.45%	18.60%
B2	0.00%	14.55%	0.00%	0.00%	19.81%	12.96%
B3	0.00%	19.09%	0.00%	0.00%	16.67%	17.45%
L4*	0.00%	20.00%	0.00%	0.00%	14.29%	23.81%

* the same

	rs7126340	rs634008	rs12294029	rs4988300	rs3867143	rs11606508
	SNP 13	SNP 14	SNP 15	SNP 16	SNP 18	SNP 20
	intronic PPP6R3 gene (3´LRP5)	intronic	intronic PPP6R3 gene (3´LRP5)	intronic	3´ intronic	intronic
LEUVEN MAFs						
Controls	24.21%	40.12%	24.31%	42.94%	15.23%	11.86%
Diverticulitis	23.53%	57.06%	23.57%	50.71%	14.58%	13.89%
IBD	25.98%	47.16%	26.29%	50.27%	14.67%	13.15%
UC	19.66%	44.02%	20.33%	46.15%	13.07%	11.67%
CD	28.07%	48.20%	28.26%	51.64%	15.21%	13.64%
male	29.46%	48.55%	29.71%	51.76%	15.45%	13.82%
female	23.28%	46.46%	23.70%	49.76%	13.93%	13.33%
A1	22.06%	50.00%	22.06%	51.47%	7.58%	11.76%
A2	23.47%	46.14%	24.08%	50.23%	13.98%	14.91%
A3	32.14%	45.89%	31.76%	49.32%	21.32%	8.22%
B1	27.86%	47.14%	27.86%	52.86%	15.44%	20.00%
B2	21.43%	41.38%	20.69%	43.97%	8.93%	7.89%
B3	28.95%	50.00%	29.83%	53.81%	16.67%	11.76%
L4*	28.57%	52.38%	28.57%	59.52%	17.50%	16.67%
ileal CD	28.20%	49.32%	28.44%	53.23%	15.63%	12.90%
male	32.42%	47.87%	32.98%	52.15%	15.93%	13.83%
female	25.00%	50.40%	25.00%	54.03%	15.38%	12.20%

A1	26.00%	50.00%	26.00%	52.00%	12.00%	12.00%
A2	27.27%	49.66%	27.55%	54.45%	14.64%	14.63%
A3	35.53%	46.34%	35.37%	48.78%	22.37%	7.50%
B1	29.55%	52.27%	29.55%	59.09%	16.67%	20.45%
B2	21.15%	42.59%	20.37%	45.37%	9.62%	8.49%
B3	29.33%	50.45%	30.28%	54.63%	16.99%	10.55%
L4*	28.57%	52.38%	28.57%	59.52%	17.50%	16.67%
* the same						

	rs61889560	CM053969	rs312781	rs599083	rs12226585	rs2137328
	SNP 21	SNP 22	SNP 23	SNP 24	SNP 25	SNP 26
	exonic Q-->R	exonic S-->L	intronic	intronic	intronic PPP6R3 gene (3´LRP5)	intronic upstream 5´
LEUVEN MAFs						
Controls	1.38%	0.00%	26.19%	27.27%	30.68%	41.55%
Diverticulitis	20.69%	0.00%	29.58%	33.80%	35.71%	38.71%
IBD	2.93%	0.00%	30.03%	31.67%	35.79%	37.66%
UC	4.26%	0.00%	29.89%	23.63%	26.37%	40.91%
CD	2.48%	0.00%	30.07%	34.39%	38.91%	36.54%
male	1.72%	0.00%	31.58%	34.52%	39.35%	33.96%
female	3.69%	0.00%	28.54%	30.58%	33.73%	39.64%
A1	0.00%	0.00%	35.29%	33.33%	33.82%	32.93%
A2	3.13%	0.00%	28.54%	30.75%	34.17%	39.34%
A3	1.33%	0.00%	33.11%	31.08%	38.36%	37.89%
B1	0.71%	0.00%	27.14%	35.00%	40.00%	35.87%
B2	3.33%	0.00%	23.73%	31.25%	35.34%	47.37%
B3	2.87%	0.00%	34.32%	34.21%	38.56%	33.00%
L4*	0.00%	0.00%	35.71%	38.10%	40.48%	45.00%
ileal CD	2.47%	0.00%	32.11%	35.92%	39.86%	36.28%
male	1.05%	0.00%	34.41%	39.13%	43.48%	33.91%
female	3.52%	0.00%	30.40%	33.47%	37.20%	37.96%
A1	0.00%	0.00%	38.00%	45.83%	44.00%	22.22%
A2	3.62%	0.00%	31.63%	33.92%	38.10%	37.70%
A3	0.00%	0.00%	30.49%	40.24%	47.50%	37.50%
B1	0.00%	0.00%	35.23%	43.18%	45.45%	30.00%
B2	3.57%	0.00%	24.55%	32.69%	36.11%	45.65%
B3	2.68%	0.00%	35.19%	34.43%	38.43%	35.36%
L4*	0.00%	0.00%	35.71%	38.10%	40.48%	45.00%
* the same						

	rs2306862	rs17149104	rs12416761	rs2277268	rs41494349	rs491347
	SNP 27	SNP 28	SNP13	SNP6 non-coding	SNP12	SNP26
	exonic N-->N	exonic D-->D	LRP5 Int. 17	LRP5 Ex. 9	LRP5 Ex. 2 missense	LRP5 Int. 7
LEUVEN MAFs						
Controls	13.44%	2.05%	6.83%	5.18%	1.02%	19.59%
Diverticulitis	14.79%	2.14%	6.82%	4.76%	1.14%	24.14%
IBD	15.75%	1.81%	4.52%	2.73%	0.44%	27.00%

UC	12.37%	0.57%	4.35%	2.91%	0.56%	19.44%
CD	16.91%	2.21%	4.58%	2.66%	0.40%	29.76%
male	16.27%	2.11%	6.25%	4.00%	0.48%	31.86%
female	15.17%	1.92%	3.40%	1.74%	0.34%	28.28%
A1	10.29%	4.41%	5.00%	5.00%	2.50%	37.50%
A2	15.37%	1.40%	5.14%	2.66%	0.28%	28.45%
A3	19.44%	2.08%	3.00%	2.04%	0.00%	32.98%
B1	17.14%	2.14%	3.17%	0.00%	0.00%	33.87%
B2	12.07%	1.72%	5.17%	4.31%	0.00%	25.42%
B3	17.83%	1.74%	5.56%	3.54%	0.84%	27.88%
L4*	19.05%	7.14%	6.25%	3.13%	0.00%	40.00%
ileal CD	17.76%	1.87%	4.69%	2.81%	0.39%	29.96%
male	18.68%	2.75%	6.59%	4.55%	0.00%	32.22%
female	17.07%	1.22%	3.57%	1.83%	0.00%	25.91%
A1	14.00%	4.00%	5.88%	5.88%	0.00%	38.24%
A2	17.47%	1.39%	5.67%	3.10%	0.00%	28.86%
A3	22.37%	2.50%	3.85%	2.63%	0.00%	35.14%
B1	20.45%	2.27%	3.75%	0.00%	0.00%	40.00%
B2	12.96%	0.93%	5.56%	4.63%	0.00%	26.36%
B3	18.10%	1.90%	6.25%	4.00%	0.00%	26.50%
L4*	19.05%	7.14%	6.25%	3.13%	0.00%	40.00%
* the same						

	rs4988322	rs4988331	rs682429	rs901824	rs11607268	rs12417014
	SNP11	SNP20	SNP23	SNP8	SNP38	SNP 47
	LRP5 Int. 10	LRP5 Int. 1	LRP5 5´	LRP5 Intron 20	LRP5 E16	LRP5 I17
LEUVEN			regulatory?		missense	intronic
MAFs						
Controls	7.53%	8.47%	36.67%	6.99%	0.00%	4.34%
Diverticulitis	6.82%	11.36%	31.25%	8.52%		
IBD	4.40%	7.65%	30.35%	3.44%	0.00%	4.47%
UC	5.00%	6.11%	32.22%	3.45%	0.00%	4.32%
CD	4.18%	8.20%	29.68%	3.43%	0.00%	4.51%
male	6.19%	7.21%	30.29%	5.26%	0.00%	6.42%
female	2.74%	8.90%	29.25%	2.17%	0.00%	3.18%
A1	5.00%	12.50%	30.95%	5.00%	0.00%	8.70%
A2	4.57%	8.05%	30.29%	3.13%	0.00%	4.60%
A3	3.00%	8.00%	27.55%	4.26%	0.00%	3.92%
B1	2.42%	8.87%	27.78%	0.88%	0.00%	5.47%
B2	5.08%	8.62%	32.20%	4.46%	0.00%	4.92%
B3	5.13%	8.55%	29.74%	3.27%	0.00%	4.82%
L4*	3.33%	3.33%	18.75%	3.33%	0.00%	5.26%
ileal CD	8.21%	30.92%	29.49%	3.57%	0.00%	5.40%
male	6.52%	7.14%	30.77%	5.42%	0.00%	7.14%
female	3.13%	10.71%	29.02%	0.95%	0.00%	3.91%
A1	5.88%	14.71%	33.33%	5.88%	0.00%	10.00%
A2	5.00%	8.05%	28.33%	3.68%	0.00%	5.07%
A3	3.85%	7.69%	30.26%	4.17%	0.00%	5.00%

B1	2.56%	10.26%	26.25%	1.43%	0.00%	6.98%
B2	5.45%	9.26%	31.82%	4.81%	0.00%	5.26%
B3	5.77%	8.17%	28.64%	3.19%	0.00%	5.50%
L4*	3.33%	3.33%	18.75%	3.33%	0.00%	5.26%

* the same

	SNP32	SNP41	SNP48	SNP46	SNP35	SNP38	SNP33
	LRP5 I11	LRP5	LRP5 E9	LRP5 E8	LRP5 I14	LRP5 I2	LRP5 I7
LEUVEN MAFs	intronic	intronic		non coding	intronic	intronic	intronic
Controls	6.02%	29.09%	2.82%	5.16%	21.91%	30.71%	16.49%
Diverticulitis							
IBD	4.83%	41.71%	1.69%	9.60%	30.30%	42.57%	26.69%
UC	6.02%	35.37%	1.28%	4.27%	22.84%	39.29%	18.24%
CD	4.46%	43.66%	1.81%	11.24%	32.73%	43.63%	29.37%
male	6.42%	45.87%	2.02%	10.65%	36.50%	46.73%	30.56%
female	3.13%	42.14%	1.68%	11.64%	30.20%	41.45%	28.57%
A1	8.70%	52.17%	0.00%	15.91%	47.37%	54.35%	36.96%
A2	4.29%	42.24%	1.82%	10.98%	30.37%	42.51%	27.71%
A3	3.85%	46.08%	1.16%	9.62%	35.71%	46.00%	32.69%
B1	4.62%	43.75%	0.82%	13.08%	35.00%	44.26%	32.58%
B2	4.92%	42.50%	1.75%	12.28%	32.41%	43.86%	28.33%
B3	4.78%	43.48%	1.43%	9.05%	30.56%	43.36%	26.32%
L4*	2.63%	44.44%	2.63%	13.16%	38.24%	50.00%	37.50%
ileal CD	5.12%	45.77%	1.53%	11.03%	34.00%	45.95%	29.91%
male	7.14%	47.25%	2.47%	10.56%	37.50%	47.80%	30.56%
female	4.20%	44.54%	1.36%	11.76%	29.55%	43.69%	27.50%
A1	10.00%	52.50%	0.00%	17.50%	50.00%	55.00%	37.50%
A2	4.70%	44.93%	1.43%	10.47%	31.07%	45.17%	28.04%
A3	4.88%	46.25%	1.56%	11.25%	40.79%	46.25%	35.37%
B1	5.81%	51.19%	1.22%	15.91%	42.50%	52.38%	37.50%
B2	5.26%	43.86%	1.85%	12.04%	33.33%	43.75%	29.46%
B3	5.39%	43.56%	0.55%	7.84%	29.69%	43.43%	24.75%
L4*	2.63%	44.44%	2.63%	13.16%	38.24%	50.00%	37.50%

* the same

Vienna

	rs314756	rs676318	rs556442	rs312788	rs1127291	rs638051
	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	SNP 6
	intronic	intronic	exonic V-->V	intronic	exonic A-->V	intronic
VIENNA MAFs						
Controls	13.94%	9.24%	41.63%	43.43%	1.34%	39.63%
IBD	6.32%	8.31%	31.86%	39.42%	1.78%	39.40%
UC	6.99%	10.33%	35.16%	43.24%	2.42%	43.48%
CD	5.85%	6.90%	29.54%	36.74%	1.33%	36.62%
male	5.04%	8.62%	29.57%	38.56%	0.85%	40.34%
female	6.44%	4.55%	26.54%	32.20%	1.89%	32.12%

A1	4.17%	6.25%	27.08%	35.42%	0.00%	36.00%
A2	5.66%	6.97%	29.85%	37.44%	1.42%	36.74%
A3	8.62%	6.90%	29.31%	32.76%	1.72%	36.21%
B1	3.30%	6.31%	27.18%	35.71%	0.94%	40.57%
B2	12.50%	3.13%	25.00%	33.33%	0.00%	27.08%
B3	5.09%	8.41%	33.33%	38.43%	2.34%	35.71%
L4*	3.97%	13.71%	30.65%	38.10%	1.59%	35.16%
ileal CD	5.39%	6.44%	30.81%	37.01%	1.72%	36.71%
male	4.35%	8.33%	32.39%	39.67%	1.10%	41.76%
female	6.00%	3.50%	26.02%	31.00%	2.50%	30.95%
A1	5.26%	5.26%	26.32%	34.21%	0.00%	32.50%
A2	5.99%	6.44%	31.90%	37.95%	1.81%	36.83%
A3	10.00%	7.50%	37.50%	45.00%	2.50%	45.00%
B1	2.24%	6.82%	32.03%	39.55%	1.49%	44.03%
B2	10.23%	3.41%	25.00%	31.82%	0.00%	28.41%
B3	5.43%	6.59%	32.02%	36.96%	2.75%	34.74%
L4*	3.97%	13.71%	30.65%	38.10%	1.59%	35.16%

* the same

	rs121908673	rs3736228	rs78219242	rs61370283	rs4988320	rs4988319
	SNP 7	SNP 8	SNP 9	SNP 10	SNP 11	SNP 12
	exonic T-->I	exon A-->V	exon L-->R	exon L-->M	intronic	intronic
VIENNA MAFs						
Controls	0.00%	21.70%	0.00%	0.00%	16.06%	13.22%
IBD	0.00%	13.48%	0.00%	0.00%	16.44%	14.92%
UC	0.00%	15.45%	0.00%	0.00%	18.58%	14.75%
CD	0.00%	12.17%	0.00%	0.00%	14.94%	15.04%
male	0.00%	12.08%	0.00%	0.00%	17.52%	15.55%
female	0.00%	12.87%	0.00%	0.00%	11.54%	12.78%
A1	0.00%	16.67%	0.00%	0.00%	8.33%	25.00%
A2	0.00%	11.92%	0.00%	0.00%	15.87%	14.95%
A3	0.00%	10.34%	0.00%	0.00%	13.79%	7.14%
B1	0.00%	8.02%	0.00%	0.00%	15.87%	15.87%
B2	0.00%	10.42%	0.00%	0.00%	13.83%	8.51%
B3	0.00%	16.36%	0.00%	0.00%	14.95%	15.63%
L4*	0.00%	8.73%	0.00%	0.00%	13.93%	14.29%
ileal CD	0.00%	12.62%	0.00%	0.00%	15.17%	14.95%
male	0.00%	13.44%	0.00%	0.00%	18.33%	16.48%
female	0.00%	12.50%	0.00%	0.00%	11.62%	10.89%
A1	0.00%	18.42%	0.00%	0.00%	7.89%	23.68%
A2	0.00%	11.45%	0.00%	0.00%	16.16%	14.67%
A3	0.00%	12.50%	0.00%	0.00%	20.00%	10.53%
B1	0.00%	8.82%	0.00%	0.00%	17.42%	17.69%
B2	0.00%	9.09%	0.00%	0.00%	13.95%	9.30%
B3	0.00%	17.20%	0.00%	0.00%	13.74%	15.26%
L4*	0.00%	8.73%	0.00%	0.00%	13.93%	14.29%

* the same

	rs7126340	rs634008	rs12294029	rs4988300	rs3867143	rs11606508
	SNP 13	SNP 14	SNP 15	SNP 16	SNP 18	SNP 20
	intronic PPP6R3 Gen (3´LRP5)	intronic	intronic PPP6R3 Gen (3´LRP5)	intronic	3´ intronic	intronic
VIENNA MAFs						
Controls	26.85%	50.97%	27.59%	51.96%	14.26%	14.08%
IBD	24.66%	45.87%	24.94%	47.54%	12.10%	13.28%
UC	27.03%	49.46%	27.39%	50.54%	14.40%	14.86%
CD	22.99%	43.32%	23.19%	45.44%	10.47%	12.17%
male	21.98%	41.81%	22.22%	44.44%	9.65%	11.11%
female	21.76%	42.42%	21.97%	43.56%	10.38%	11.74%
A1	18.75%	39.58%	18.75%	39.58%	14.58%	6.25%
A2	24.04%	44.02%	24.29%	46.43%	10.44%	12.62%
A3	18.97%	41.38%	18.97%	43.10%	7.14%	13.79%
B1	21.36%	41.83%	21.63%	43.75%	8.25%	13.94%
B2	22.92%	45.83%	22.92%	48.96%	11.70%	11.46%
B3	25.70%	42.52%	25.93%	44.44%	11.43%	11.57%
L4*	16.39%	45.97%	17.46%	48.41%	7.38%	13.49%
ileal CD	24.88%	43.07%	25.12%	45.57%	10.10%	13.30%
male	26.11%	42.78%	26.37%	46.15%	11.36%	13.19%
female	22.22%	41.00%	22.50%	42.00%	8.16%	11.50%
A1	18.42%	36.84%	18.42%	36.84%	15.79%	7.89%
A2	25.15%	44.21%	25.45%	46.97%	10.25%	13.64%
A3	25.00%	55.00%	25.00%	55.00%	7.89%	15.00%
B1	26.15%	43.18%	26.52%	45.45%	7.69%	17.42%
B2	22.73%	45.45%	22.73%	48.86%	10.47%	12.50%
B3	25.27%	41.21%	25.54%	43.48%	11.80%	10.33%
L4*	16.39%	45.97%	17.46%	48.41%	7.38%	13.49%
* the same						

	rs61889560	CM053969	rs312781	rs599083	rs12226585	rs2137328
	SNP 21	SNP 22	SNP 23	SNP 24	SNP 25	SNP 26
	exonic Q-->R	exonic S-->L	intronic	intronic	intronic PPP6R3 Gen (3´LRP5)	intronic vor 5´
VIENNA MAFs						
Controls	11.32%	0.00%	31.40%	33.25%	37.06%	35.20%
IBD	2.41%	0.00%	25.67%	30.09%	33.45%	37.96%
UC	0.82%	0.00%	27.15%	33.88%	38.25%	32.38%
CD	3.49%	0.00%	24.62%	27.41%	30.08%	41.62%
male	3.31%	0.00%	23.71%	26.32%	31.20%	43.83%
female	4.01%	0.00%	22.73%	25.95%	25.95%	42.13%
A1	4.00%	0.00%	29.17%	27.08%	25.00%	50.00%
A2	3.90%	0.00%	24.40%	27.54%	31.25%	41.40%
A3	0.00%	0.00%	22.41%	26.79%	25.86%	36.49%
B1	1.89%	0.00%	21.63%	24.76%	28.16%	44.37%
B2	1.04%	0.00%	26.04%	25.00%	26.04%	44.12%
B3	6.09%	0.00%	26.64%	30.95%	34.11%	37.50%

L4*	3.08%	0.00%	23.39%	27.05%	29.84%	50.00%
ileal CD	3.57%	0.00%	24.50%	28.75%	31.75%	39.66%
male	2.15%	0.00%	23.89%	29.55%	35.56%	39.47%
female	5.24%	0.00%	21.00%	25.50%	25.25%	42.22%
A1	5.00%	0.00%	26.32%	26.32%	23.68%	52.17%
A2	3.24%	0.00%	24.09%	29.01%	32.30%	38.21%
A3	0.00%	0.00%	30.00%	34.21%	32.50%	30.00%
B1	1.49%	0.00%	22.73%	29.55%	33.85%	42.13%
B2	1.14%	0.00%	25.00%	25.00%	26.14%	43.55%
B3	6.12%	0.00%	25.27%	29.78%	32.22%	35.78%
L4*	3.08%	0.00%	23.39%	27.05%	29.84%	50.00%
* the same						

	rs2306862	rs17149104	rs12416761	rs2277268	rs41494349	rs491347
	SNP 27	SNP 28	SNP13	SNP6	SNP12	SNP26
	exonic N-->N	exonic D-->D	LRP5 Int. 17	LRP5 Ex. 9	LRP5 Ex. 2	LRP5 Int. 7
VIENNA MAFs				non coding	missense	
Controls	15.61%	1.80%	5.72%	5.67%	0.36%	27.64%
IBD	13.53%	2.67%	6.92%	6.49%	0.70%	22.87%
UC	15.59%	4.24%	6.21%	4.94%	0.93%	26.56%
CD	12.07%	1.57%	7.36%	7.44%	0.57%	20.61%
male	11.21%	0.88%	6.91%	7.72%	0.00%	20.25%
female	11.83%	1.95%	6.57%	6.34%	1.10%	19.85%
A1	16.67%	0.00%	4.17%	2.27%	0.00%	27.27%
A2	11.54%	1.73%	7.89%	7.93%	0.48%	20.43%
A3	12.07%	1.79%	6.25%	7.81%	1.56%	17.19%
B1	9.62%	1.46%	6.67%	6.80%	0.94%	18.10%
B2	13.54%	0.00%	8.33%	7.29%	1.04%	17.71%
B3	12.74%	2.45%	7.55%	8.10%	0.00%	23.30%
L4*	6.56%	1.67%	4.76%	6.56%	0.00%	21.77%
ileal CD	12.44%	2.05%	8.05%	7.67%	0.49%	21.29%
male	13.33%	1.15%	7.37%	7.89%	0.00%	23.12%
female	10.10%	2.58%	7.62%	6.86%	0.96%	18.27%
A1	15.79%	0.00%	5.26%	2.94%	0.00%	29.41%
A2	12.27%	2.20%	8.79%	8.23%	0.30%	20.73%
A3	15.00%	2.50%	4.76%	7.14%	2.38%	19.05%
B1	10.61%	2.31%	8.21%	7.69%	0.75%	20.90%
B2	12.50%	0.00%	9.09%	7.95%	1.14%	17.05%
B3	13.89%	2.87%	7.53%	7.61%	0.00%	23.33%
L4*	6.56%	1.67%	4.76%	6.56%	0.00%	21.77%
* the same						

	rs4988322	rs4988331	rs682429	rs901824	rs11607268	rs12417014
	SNP11	SNP20	SNP23	SNP8	SNP38	SNP 47
	LRP5 Int. 10	LRP5 Int. 1	LRP5 5´	LRP5 Intron 20	LRP5 E16	LRP5 I17
VIENNA MAFs			regulatory		missense	intronic
Controls	6.18%	7.36%	28.64%	7.47%	0.16%	5.27%
IBD	27.78%	9.53%	31.57%	8.27%	0.00%	6.98%
UC	27.59%	10.25%	34.26%	6.96%	0.00%	6.00%
CD	27.85%	9.09%	29.92%	9.07%	0.00%	7.37%
male	25.00%	11.79%	29.27%	7.92%	0.00%	7.56%
female	29.17%	6.25%	31.62%	8.58%	0.00%	6.06%
A1	50.00%	10.87%	32.61%	4.55%	0.00%	6.25%
A2	27.27%	8.85%	30.14%	9.95%	0.00%	6.86%
A3	25.00%	9.38%	26.56%	6.45%	0.00%	10.00%
B1	30.00%	7.14%	28.10%	9.31%	0.00%	6.49%
B2	30.77%	6.25%	32.29%	9.38%	0.00%	6.94%
B3	25.00%	12.86%	30.95%	8.74%	0.00%	8.33%
L4*	29.41%	7.26%	27.42%	5.93%	0.00%	3.19%
ileal CD	28.57%	8.58%	32.11%	10.05%	0.00%	7.14%
male	25.00%	10.00%	31.58%	8.70%	0.00%	7.35%
female	30.00%	7.21%	33.65%	9.80%	0.00%	6.58%
A1	50.00%	13.89%	38.89%	5.88%	0.00%	7.69%
A2	27.78%	7.88%	32.42%	11.11%	0.00%	6.61%
A3	25.00%	9.52%	23.81%	5.00%	0.00%	11.54%
B1	31.82%	4.48%	31.34%	11.72%	0.00%	6.25%
B2	30.77%	6.82%	32.95%	10.23%	0.00%	7.58%
B3	25.00%	12.50%	32.61%	8.89%	0.00%	7.69%
L4*	29.41%	7.26%	27.42%	5.93%	0.00%	3.19%
* the same						

	rs2242339	rs312778	rs4988321	rs545382	rs554734	rs643981	rs686921
	SNP32	SNP41	SNP48	SNP46	SNP35	SNP38	SNP33
	LRP5 I11	LRP5	LRP5 E9	LRP5 E8	LRP5 I14	LRP5 I2	LRP5 I7
VIENNA MAFs	intronic	intronic		non coding	intronic	intronic	intronic
Controls	6.91%	44.17%	3.43%	8.39%	31.29%	42.94%	26.94%
IBD	9.75%	36.94%	4.98%	8.62%	29.38%	35.63%	22.30%
UC	6.25%	40.13%	5.88%	9.74%	31.88%	39.58%	25.68%
CD	11.05%	35.68%	4.64%	8.22%	28.46%	34.07%	21.03%
male	12.82%	35.47%	3.57%	10.00%	29.17%	34.15%	21.35%
female	7.39%	33.50%	5.38%	6.25%	25.00%	32.11%	20.00%
A1	9.38%	40.63%	6.67%	18.75%	34.38%	40.00%	28.13%
A2	10.74%	34.74%	5.14%	7.27%	28.81%	33.33%	21.84%
A3	12.50%	38.10%	0.00%	8.33%	20.00%	34.21%	10.00%
B1	9.42%	35.71%	2.00%	7.83%	23.33%	34.67%	17.09%
B2	12.07%	32.43%	6.06%	4.55%	26.39%	27.42%	20.00%
B3	11.59%	35.42%	7.14%	10.00%	32.39%	34.29%	24.67%
L4*	5.95%	36.46%	2.33%	14.29%	28.72%	35.23%	22.83%

ileal CD	10.53%	36.58%	5.67%	8.84%	30.21%	35.00%	23.15%
male	11.48%	36.96%	4.48%	11.33%	32.58%	35.16%	25.36%
female	8.09%	33.55%	6.43%	5.88%	25.00%	32.88%	19.74%
A1	11.54%	39.29%	7.69%	19.23%	38.46%	37.50%	30.77%
A2	10.19%	34.43%	6.09%	7.78%	30.25%	33.33%	23.39%
A3	12.50%	53.85%	0.00%	9.38%	20.83%	50.00%	12.50%
B1	8.89%	41.84%	3.19%	10.78%	28.26%	40.43%	21.43%
B2	13.46%	32.35%	5.00%	4.88%	27.27%	26.79%	20.31%
B3	10.66%	33.85%	7.94%	9.15%	32.03%	33.59%	25.37%
L4*	5.95%	36.46%	2.33%	14.29%	28.72%	35.23%	22.83%

* the same

Stuttgart

	rs314756	rs676318	rs556442	rs312788	rs1127291	rs638051
	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	SNP 6
	intronic	intronic	exonic V-->V	intronic	exonic A-->V	intronic
STUTTGART MAFs						
Controls	8.45%	6.74%	29.14%	42.55%	1.41%	41.37%
C Indeterminata	6.25%	10.42%	29.17%	43.75%	2.08%	47.92%
Inflammatory C	11.90%	9.52%	38.64%	45.24%	0.00%	50.00%
IBD	8.38%	6.13%	32.49%	39.94%	0.75%	37.09%
UC	7.30%	6.67%	33.46%	39.39%	1.09%	37.13%
CD	9.14%	5.76%	31.82%	40.31%	0.51%	37.06%
male	6.77%	6.45%	35.05%	38.17%	0.52%	39.58%
female	11.34%	5.32%	29.90%	43.09%	0.52%	35.05%
A1	3.03%	1.52%	25.76%	37.88%	1.52%	41.18%
A2	9.92%	6.47%	34.02%	39.22%	0.41%	33.75%
A3	17.39%	11.36%	39.13%	50.00%	0.00%	39.13%
B1	6.34%	7.14%	31.94%	34.29%	1.41%	32.39%
B2	13.64%	5.56%	29.10%	45.24%	0.76%	37.69%
B3	5.77%	8.00%	39.42%	38.00%	0.00%	43.27%
L4*	4.55%	13.64%	54.55%	45.45%	9.09%	50.00%
ileal CD	10.06%	5.88%	30.56%	38.24%	0.31%	35.22%
male	7.69%	6.67%	33.13%	35.33%	0.00%	37.82%
female	12.34%	5.41%	29.49%	41.89%	0.66%	33.12%
A1	3.85%	1.92%	27.78%	36.54%	0.00%	38.89%
A2	10.50%	6.84%	33.82%	38.42%	0.50%	33.84%
A3	20.59%	9.38%	35.29%	46.88%	0.00%	32.35%
B1	6.12%	4.17%	26.00%	29.17%	0.00%	28.00%
B2	14.17%	6.14%	28.69%	43.86%	0.00%	36.44%
B3	7.89%	9.72%	39.74%	34.72%	0.00%	39.47%
L4*	4.55%	13.64%	54.55%	45.45%	9.09%	50.00%

* the same

	rs121908673	rs3736228	rs78219242	rs61370283	rs4988320	rs4988319
	SNP 7	SNP 8	SNP 9	SNP 10	SNp 11	SNP 12
	exonic T-->I	exon A-->V	exon L-->R	exon L-->M	intronic	intronic
STUTTGART MAFs						
Controls	0.00%	14.89%	0.00%	0.00%	18.09%	13.48%
C Indeterminata	0.00%	6.25%	0.00%	0.00%	16.67%	16.67%
Inflammatory C	0.00%	22.73%	0.00%	0.00%	18.18%	18.18%
IBD	0.00%	17.60%	0.00%	0.00%	13.80%	16.91%
UC	0.00%	19.78%	0.00%	0.00%	14.75%	17.50%
CD	0.00%	16.08%	0.00%	0.00%	13.13%	16.50%
male	0.00%	17.35%	0.00%	0.00%	13.92%	17.35%
female	0.00%	15.46%	0.00%	0.00%	12.89%	15.79%
A1	0.00%	12.12%	0.00%	0.00%	17.65%	15.15%
A2	0.00%	16.67%	0.00%	0.00%	11.48%	16.39%
A3	0.00%	21.74%	0.00%	0.00%	18.18%	13.64%
B1	0.00%	13.19%	0.00%	0.00%	9.72%	15.97%
B2	0.00%	15.67%	0.00%	0.00%	15.38%	16.67%
B3	0.00%	19.23%	0.00%	0.00%	15.09%	17.65%
L4*	0.00%	9.09%	0.00%	0.00%	22.73%	22.73%
ileal CD	0.00%	14.81%	0.00%	0.00%	12.81%	15.31%
male	0.00%	15.63%	0.00%	0.00%	13.92%	15.63%
female	0.00%	14.74%	0.00%	0.00%	12.34%	15.13%
A1	0.00%	12.96%	0.00%	0.00%	16.67%	15.38%
A2	0.00%	16.18%	0.00%	0.00%	11.39%	16.34%
A3	0.00%	20.59%	0.00%	0.00%	15.63%	11.76%
B1	0.00%	12.00%	0.00%	0.00%	9.00%	13.00%
B2	0.00%	14.75%	0.00%	0.00%	15.25%	15.57%
B3	0.00%	17.95%	0.00%	0.00%	11.54%	16.22%
L4*	0.00%	9.09%	0.00%	0.00%	22.73%	22.73%
* the same						

	rs7126340	rs634008	rs12294029	rs4988300	rs3867143	rs11606508
	SNP 13	SNP 14	SNP 15	SNP 16	SNP 18	SNP 20
	intronic PPP6R3 Gen (3´LRP5)	intronic	intronic PPP6R3 gene (3´LRP5)	intronic	3´ intronic	intronic
STUTTGART MAFs						
Controls	21.69%	47.86%	22.30%	50.35%	11.15%	13.67%
C Indeterminata	20.83%	41.67%	20.83%	41.67%	8.33%	8.33%
Inflammatory C	26.19%	54.76%	26.19%	61.90%	17.50%	16.67%
IBD	25.31%	48.95%	24.77%	50.31%	13.91%	14.42%
UC	24.62%	53.28%	24.06%	54.14%	15.53%	14.55%
CD	25.80%	45.90%	25.26%	47.63%	12.77%	14.32%
male	26.63%	43.62%	26.88%	45.16%	14.52%	17.02%
female	26.09%	48.45%	24.73%	50.54%	11.54%	11.70%
A1	25.76%	42.65%	24.24%	43.75%	9.38%	10.61%
A2	26.32%	45.76%	25.65%	46.55%	12.61%	14.53%

A3	23.81%	47.83%	25.00%	52.27%	16.67%	9.09%
B1	26.43%	44.44%	26.43%	45.71%	10.87%	12.14%
B2	21.31%	46.88%	22.22%	48.41%	12.10%	13.49%
B3	29.00%	48.08%	26.00%	53.06%	14.29%	18.63%
L4*	36.36%	50.00%	36.36%	54.55%	9.09%	9.09%
ileal CD	23.84%	43.99%	23.36%	45.75%	11.44%	13.64%
male	24.32%	39.61%	24.67%	42.00%	12.67%	15.13%
female	24.66%	48.70%	23.29%	50.00%	10.81%	12.16%
A1	25.00%	44.44%	25.00%	44.23%	9.62%	7.69%
A2	25.27%	43.37%	24.47%	44.74%	12.11%	14.58%
A3	21.88%	50.00%	21.88%	53.13%	15.63%	6.25%
B1	23.96%	40.00%	23.96%	40.63%	9.38%	10.42%
B2	19.64%	47.41%	20.18%	48.25%	11.40%	14.04%
B3	26.39%	44.74%	23.61%	48.61%	12.50%	17.57%
L4*	36.36%	50.00%	36.36%	54.55%	9.09%	9.09%

* the same

	rs61889560	CM053969	rs312781	rs599083	rs12226585	rs2137328
	SNP 21	SNP 22	SNP 23	SNP 24	SNP 25	SNP 26
	exonic Q-->R	exonic S-->L	intronic	intronic	intronic PPP6R3 gene (3´LRP5)	intronic upstream 5´
STUTTGART MAFs						
Controls	1.79%	0.00%	27.50%	25.54%	28.62%	34.55%
C Indeterminata	0.00%	0.00%	28.26%	27.08%	31.25%	42.19%
Inflammatory C	4.55%	0.00%	38.10%	35.71%	38.10%	33.33%
IBD	4.42%	0.00%	28.53%	28.09%	31.21%	40.17%
UC	4.32%	0.00%	27.94%	27.82%	31.06%	33.33%
CD	4.50%	0.00%	28.95%	28.27%	31.32%	44.42%
male	4.59%	0.00%	26.88%	31.18%	33.51%	48.09%
female	4.59%	0.00%	31.18%	26.60%	30.43%	39.34%
A1	2.94%	0.00%	20.31%	24.24%	27.27%	52.04%
A2	5.28%	0.00%	29.31%	29.31%	32.33%	43.27%
A3	4.35%	0.00%	34.09%	36.36%	35.71%	46.55%
B1	2.08%	0.00%	26.43%	29.29%	33.57%	38.33%
B2	6.72%	0.00%	33.33%	24.60%	27.78%	50.54%
B3	5.66%	0.00%	26.53%	36.00%	35.00%	45.83%
L4*	0.00%	0.00%	36.36%	50.00%	50.00%	40.00%
ileal CD	5.56%	0.00%	27.78%	26.14%	29.41%	46.96%
male	5.63%	0.00%	25.33%	28.67%	31.58%	52.70%
female	5.77%	0.00%	30.41%	25.00%	28.77%	39.06%
A1	3.70%	0.00%	21.15%	25.00%	26.92%	56.25%
A2	6.37%	0.00%	29.47%	28.95%	31.58%	45.80%
A3	5.88%	0.00%	31.25%	28.13%	31.25%	42.86%
B1	3.00%	0.00%	21.88%	22.92%	28.13%	42.19%
B2	7.38%	0.00%	31.58%	22.81%	26.72%	51.18%
B3	7.69%	0.00%	25.00%	34.72%	33.33%	50.00%
L4*	0.00%	0.00%	36.36%	50.00%	50.00%	40.00%

* the same

	rs2306862	rs17149104
	SNP 27	SNP 28
	exonic N-->N	exonic D-->D
STUTTGART MAFs		
Controls	11.43%	3.85%
C Indeterminata	6.25%	4.35%
Inflammatory C	21.43%	0.00%
IBD	13.62%	2.17%
UC	13.16%	4.17%
CD	13.95%	0.84%
male	14.13%	1.14%
female	14.36%	0.57%
A1	12.12%	1.56%
A2	13.48%	0.93%
A3	18.18%	0.00%
B1	13.57%	2.24%
B2	11.11%	0.85%
B3	17.00%	0.00%
L4*	13.64%	10.00%
ileal CD	11.76%	0.69%
male	11.33%	0.70%
female	12.84%	0.71%
A1	11.54%	0.00%
A2	12.63%	1.12%
A3	15.63%	0.00%
B1	11.46%	1.06%
B2	9.65%	0.00%
B3	13.89%	0.00%
L4*	13.64%	10.00%
* the same		