Disease Underlying Genetics in Ileal Crohn's Disease: Disturbed Antimicrobial Defense and Impaired Wnt Signaling at the Epithelial Barrier

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Maureen Koslowski

aus Konstanz

Tübingen

2011

Tag der mündlichen Prüfung:	19.04.2012
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	PD Dr. Jan Wehkamp
2. Berichterstatter:	Prof. Dr. Andreas Peschel
3. Berichterstatter:	Prof. Dr. Thomas Seufferlein

Parts of this thesis have previously been published:

1. Genetic variants of Wnt transcription factor TCF-4 (TCF7L2) putative promotor region are associated with small intestinal Crohn's Disease

Maureen J. Koslowski, Irmgard Kübler, Mathias Chamaillard, Elke Schaeffeler, Walter Reinisch, Guoxing Wang, Julia Beisner, Alexander Teml, Laurent Peyrin-Biroulet, Stefan Winter, Klaus R. Herrlinger, Paul Rutgeerts, Séverine Vermeire, Rachel Cooney, Klaus Fellermann, Derek Jewell, Charles L. Bevins, Matthias Schwab, Eduard F. Stange and Jan Wehkamp, 2009, PLoSone *4*, e4496.

2. Method for determining the predisposition for Crohn's Disease / Verfahren zur Bestimmung der Prädisposition für Morbus Crohn.

Maureen J. Koslowski, Eduard F. Stange and Jan Wehkamp; Patent: WO002010072389A1 / DE102008064509A1**.** International Application No.: PCT/EP2009/009176 Publication Date: 01.07.2010 International Filing Date: 21.12.2009

3. Influence of standard treatment on ileal and colonic antimicrobial Defensin expression in active Crohn's disease

Irmgard Kübler, Maureen J. Koslowski, Michael Gersemann, Klaus Fellermann, Julia Beisner, Svetlana Becker, Katja Rothfuss, Klaus R. Herrlinger, Eduard F. Stange, Jan Wehkamp. (2009). Alimentary Pharmacology & Therapeutics *30*, 621–633.

4. Innate antimicrobial host defense in small intestinal Crohn's disease

Maureen J. Koslowski, Julia Beisner, Eduard F. Stange, Jan Wehkamp. (2010). Int J Med Microbiol. *300*, 34-40.

5. Association of a functional variant in the Wnt co-receptor LRP6 with early onset ileal Crohn´s disease

Maureen J. Koslowski, Zora Teltschik, Julia Beisner, Elke Schaeffeler, Guoxing Wang, Irmgard Kuebler, Michael Gersemann, Rachel Cooney, Derek Jewell, Walter Reinisch, Séverine Vermeire, Paul Rutgeerts, Matthias Schwab Eduard F. Stange, Jan Wehkamp. (2012). PLoSGenetics 8(2), e1002523.

ACKNOWLEDGEMENT

It is customary for dissertators to reserve excessive prose and sentimentality for their acknowledgements. Given my fondness for hyperbole descriptions and interlaced sentences and my constant fight of eliminating it from my scientific work, it is my pleasure to skip the censoring when saying thanks those who made this thesis possible.

My deep gratitude goes to everybody at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, where I was able to do the experimental part of my thesis from February 2008 till May 2010, as well as to my extraordinary friends and family. I am grateful for you all but especially want to address some in the following.

First, I owe my respect to PD Dr. Jan Wehkamp, who supervised my thesis with encouraging words and lots of expertise. His enthusiastic approach on science gave me direction as well as freedom and the possibility to develop my own understanding of the subject. Even though most students would mainly acknowledge guidance and advice, I want to highlight his optimism and creativity; two things which are often rare in the daily research grind and greatly contributed to my work. I am also indebted to PD Dr. Andreas Peschel who liberally assumed my supervision with regards to the University faculty. I know this is not always an easy task with external PhD candidates. Furthermore I would like to pay tribute to the scientific intuition and experience of Prof. Dr. Eduard F. Stange. His analytical mindset and sense of humor lead to some extremely helpful comments, which were always received with a balanced mixture of gratitude (for valued improvement) and jealousy ("why didn't I think of this?").

I would like to extent my acknowledgement to the head of the IKP, Prof. Dr. Matthias Schwab for an inspiring time at the institute. I greatly benefitted from the excellent technical equipment (the Sequenom, but also: the coffee maker) and the conducive, scientific atmosphere. My sincere gratitude furthermore goes to Dr. Elke Schäffeler from Prof. Schwab's group, for introducing me to the world of SNP genotyping. Even though it first felt a bit like wonderland it soon became a quite comfortable place. I've also been fortunate to have had the help of Dr. Stefan Winter who was always willing to engage in my, at first admittedly limited, ideas of high-throughput data analysis and to invest his time (which is scarce) and his energy (which is abundant) to help me improve.

Something I truly appreciated was the cooperative mindset within the research groups of PD Dr. Wehkamp and Prof. Stange. I owe the whole defensin crew, you know who you are, a very warm thank you. I am equally grateful for the lively work discussions and the friendship, Italian dinners and all the time we shared aside. I have to highlight Jutta Bader and Kathleen Siegel, two extraordinary technicians who gave me many "*deus ex machina"* moments during my PhD candidacy and mastered the lab and its inhabitants with a calm attitude and lots of wit and wisdom. I also want to

acknowledge Zora Teltschik for being gone with the Wnts with me and co-riding a snowmobile when holding on alone was simply impossible. Svetlana Becker, it was extremely fun and supportive to share an office and many interesting conversations about the complications of cell signaling and everyday life with you. To all my other co-workers: “thanks to you, I’m much obliged for such a pleasant stay” and I am glad to have the chance to work with you again.

This thesis would furthermore not have been possible without the funding of the Robert-Bosch-Foundation and the national and international cooperation partners who contributed their expertise and biomaterial. I am deeply thankful for this support. Additionally I would like to express my gratitude to all the physicians of the Gastroenterology Department at the Robert-Bosch-Hospital for their exemplary work and support of scientific research.

Last but certainly not least I want to thank my close friends and family; you guys always had my back. I am lucky to have you all. It is impossible to mention each and every one who’d deserve it, but I’d like to thank Gaby for being a true comrade and my Sushi at the lake connection during an incredible summer. I also want to thank Silas for being my rock and for teaching me how to climb mountains, literally and figuratively. I’m excited about all our great adventures to come! My remarkable parents Karin and Roger Koslowski, simply to say thank you does not cut it. You’ve been my compass, my backbone, and without fail provided something more than just everyday support: a big smile every time we see each other.

Once I rose above the noise and confusion
just to get a glimpse beyond this illusion …

……. and if I claim to be a wise man,
well it surely means that I don't know

K. Livgren for Kansas

ABBREVIATIONS

Activator protein 1	AP-1
Adenomatous polyposis coli	APC
Antimicrobial peptide	AMP
ATG16 autophagy related 16-like 1	ATG16L1
Aqua destillata	aqua dest.
Azoxymethane	AOM
Bone mass density	BMD
Casein kinase I epsilon"	CKIε
Chronisch Entzündliche Darmerkrankungen	CED
Colitis associated cancer	CAC
Colorectal cancer	CRC
Confidence Interval	CI
Crohn's Disease	CD
Defensin, beta	DEFB
Defensin, alpha	DEFA
Dickkopf-1	Dkk1
Dishevelled	Dvl
Disks large homolog 5	DLG5
Escherichia coli	*E. coli*
Enteropathogenic Escherichia coli	EHEC
Ethylenediaminetetraacetic acid	EDTA
Fibroblast growth factor receptor 3	FGFR-3
Forkhead box L1	Foxl1
Frequently rearranged in advanced T-cell lymphomas- 1	Frat-1
Gastrointestinal tract	GIT
Genome-wide association studies	GWAS
Glycogen synthase kinase 3 β	GSK-3β
Helicobacter pylori	*H. pylori*
Hardy Weinberg	HW
Hepatocyte growth factor	HGF
Hepatocyte nuclear factor	HNF
Human-beta-defensin	HBD
Human defensin	HD
Human defensin 5 propeptide	Pro-HD5
Hydrochloric acid	HCl
Indeterminate colitis	IC
Inflammatory bowel diseases	IBD
Interleukin	IL
Intermediate conductance calcium-activated potassium channel protein	KCNN4
Leucine-rich repeat G protein-coupled receptor	LGR
Linkage disequilibrium/a	LD
Low density lipoprotein receptor-related protein	LRP
Lymphoid enhancer-binding factor	Lef
Lysogeny (also known as Luria) broth	LB
Mass spectrometry	MS
Matrix assisted laser desorption/ionization time-of-flight	MALDI-TOF
Microbe-associated molecular patterns	MAMPs
Minor allele frequency	MAF
Murmayldipeptide	MDP

Myeloid differentiation primary response gene	MyD88
Myeloid translocation gene	MTG
National Center for Biotechnology Information	NCBI
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	IkB
Nucleotide-binding oligomerization domain containing molecule	NOD
Odds ratio	OR
Paneth cell	PC
Pattern recognition receptors	PRR
Pathogen- associated molecular patterns	PAMPs
Phospholipase A2 group IIA	$sPLA_2$ or PLA2G2A
Polymerase chain reaction	PCR
Primer extension	PEX
Regenerating islet-derived 3 gamma	RegIIIγ
Reverse transcriptase	RT
Rheumatoid arthritis	RA
R-spondin	RSPO
SAM pointed domain containing ets transcription factor	Spdef
Shrimp alkaline phosphatase	SAP
Single nucleotide polymorphism	SNP
Single nucleotide polymorphism database	SNPdb
Spearmen rank	Spearman r
TCF7- like 1	TCF7L1
TCF7- like 2	TCF7L2
Toll like receptors"	TLR´s
Transcription factor (T-cell specific, high-mobility group (HMG)-box)	TCF
Trichinella spiralis	*T. spiralis*
Tris(hydroxymethyl)aminomethane	Tris
Tumor necrosis factor	TNF
Ulcerative colitis	UC
Unmethylated cytidine-phosphate-guanosine	CpG
Wildtype	WT
Wnt response element	WRE
X-box binding protein 1	XBP1

Units: *number (n), revolutions per minute (rpm), gram (g), liter (l), micro (µ), milli (m), nano (n), molar (M), base pair(s) (bp), minute (min), second (sec)*

Nucleic acids: *Deoxyribonucleic acid (DNA), copy DNA (cDNA), Ribonucleic acid (RNA), messenger RNA (mRNA), deoxynucleoside triphosphate (dNTP)*

Nucleic acid bases: *adenine (A), tyrosine (T), cytosine (C), guanine (G)*

TABLE OF CONTENTS

1. INTRODUCTION

The study of complex, non Mendelian inherited diseases presents various challenges. But without a doubt, their investigation also provides opportunities to improve our understanding of their diverse etiologies and helps in paving the way for prevention, new therapeutics, and diagnostics. Challenges lie in the necessity of a broad and well integrated research approach, but also in the demand of a close relevance to clinical findings. In the case of investigations on the chronic, reoccurring and to date incurable inflammatory bowel diseases (IBD) the picture is complicated by a not completely understood environmental influence and the still partly unclarified pathogenesis. Even though there is an ongoing dispute about basic and general disease mechanisms, a major role of an imbalanced microbial-epithelial interaction at the intestinal barrier is commonly accepted among clinicians and researchers (Stange, 2009). This imbalance has been and currently is extensively studied on various levels. The herein presented work aimed at further elucidating disease underlying disturbances on the epithelial or so to speak the “host” side in the development of IBD. Pathomechanisms which impair specifically the small intestinal epithelial barrier have been studied in particular. The following introductory paragraphs will provide an overview on the gastrointestinal system and the importance of innate defense mechanisms. They will furthermore discuss known mechanisms which underlie innate defense defects in IBD and particularly in small intestinal Crohn's Disease.

1.1 The human gastrointestinal tract

The human gastrointestinal tract (GIT) consists of an upper and a lower part with specific and important functions controlled by the nervous and endocrine systems. The former includes mouth, pharynx, esophagus and stomach and mediates ingestion and digestion of food. Subsequent absorption processes are mostly subject to the small intestine, the duodenum, jejunum, and ileum. The second part of the lower GIT, the large bowel, the colon and rectum, facilitate water and final nutrient resorption and defecation. Even though the processes taking place in the different compartments are

remarkably diverse and specialized, the complete GIT exhibits, amongst few exceptions, a fundamental structural and morphological consistency.

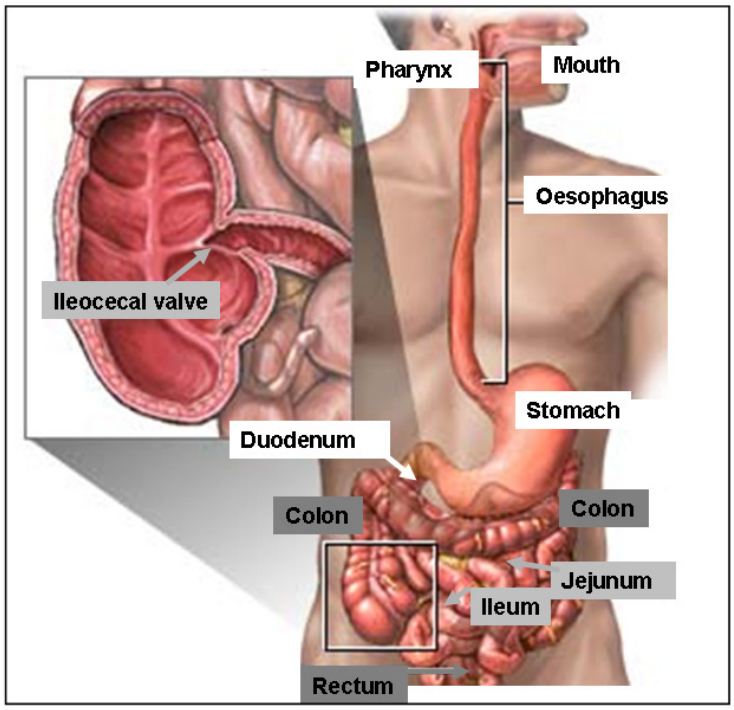

Figure 1.1 the human gastrointestinal tract (GIT); white: upper GIT, mediates ingestion and main digestion; grey highlighted: lower GIT, main tasks: absorption, resorption and defecation; (light grey: small bowel compartments; dark grey: large bowel, separated by the ileocecal valve). Adapted/modified graphic hosted on Medline sourced from A.D.A.M., Inc (now owned by Ebix, Inc) (http://www.nlm.nih.gov/medlineplus/ency/imagepages/19293.htm)

This consistency is based on a common anatomy with four basic layers or tunics: from the outermost layer inwards the tunicae serosa, muscularis, submucosa and mucosa (Tortora and Derrickson, 2008). The latter represents the lining towards the lumen, with a direct contact to its content and harbors the most variability. It endows the GIT to its specific tasks along its length. The most crucial variability of the mucosa is provided by the thin epithelial cell layer (*lamina epithelialis*) resting on top. It comprehends absorptive enterocytes and a comparatively smaller amount of secretory cells: goblet cells, which secrete mainly mucus, enteroendocrine cells, with various subtypes secreting different hormones and specifically in the small intestine the

antimicrobial peptide producing Paneth cells (PCs) (Crosnier et al., 2006; Bevins and Salzman, 2011). Beneath the lamina epithelialis but still within the tunica mucosa resides a layer - the *lamina propria* - of loose connective tissue, comprising blood vessels and lymphatic nodules, which supply the epithelium. The outermost mucosal layer of smooth muscles (*lamina muscularis mucosae*) mediates dynamic movements and folding of the mucosa. In the small intestine, multiple of such folding (*Plicae circularesas*) as well as the villi and crypts of the epithelium enlarge the surface to a total of 200-300 square meters. This has major advantages for resorptive purposes but also increases the interface for both beneficial but also harmful host- microbial interactions.

1.2 The microbiology of the human gastrointestinal tract

The GIT contains an immensely complex ecology of microorganisms with different lifestyles and capabilities. All together more than 500 distinct bacterial species are harbored in a typical person with numbers of up to 10^{13} to 10^{14} microorganisms in the digestive tract. The corresponding collective microbial genome ("microbiome") contains at least 100 times as many genes as the human genome, a fact that gave rise to the term "superorganism" (Gill et al., 2006). The four most important phyla found in the human GIT are *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* (Nuding et al., 2009). The composition and distribution of the menagerie varies with age, state of health and diet. Number and type of bacteria also dramatically vary by region. The stomach and proximal small intestine contain only few microorganisms due to the bactericidal gastric acid. Patients with achlorhydria or hypochlorhydria, conditions with absent or low gastric acid production, may consequently suffer from bacterial overgrowth and subsequent inflammatory or malignant complications (Friis-Hansen, 2006; Husebye, 2005; Naylor and Axon, 2003). In sharp contrast to the almost germfree conditions of the stomach and in part also the upper small intestine, stands the colonization of the colon. This part of the GIT is literally teemed with predominantly anaerobic bacteria (Sears, 2005). A transitional zone, usually in the ileum contains only moderate numbers of aerobic and anaerobic bacteria. The

colonization of the first sterile GIT starts in the small intestine and progresses until a stable mature flora is established in the first week of life (Fanaro et al., 2003). A role of this flora in the development of a proper intestinal morphology and function was already accepted in the 1970s (Thompson and Trexler, 1971) based on evidence from different germ-free or gnotobiotic animal models. An influence of bacteria has also been implicated in cell renewal rates and the development of Peyer's patches (ileum specific immune tissue) and other gut-associated lymphoid tissue (Duan et al., 2007; Heitman et al., 1980; Yamanaka et al., 2003). Besides such health-promoting effects, additional beneficial properties of certain bacteria include the improvement of digestion, absorption, vitamin synthesis and the inhibition of pathogen growth (Saulnier et al., 2009). Nonetheless, bacteria can also have rather pathogenic or detrimental properties, like the production of carcinogens or toxins, induction of diarrhea or constipation and infection. The GIT represents the largest surface in humans challenged by such harmful events and is as mentioned colonized by a remarkable community of commensals. It is therefore surprising that intestinal infections or bacterial translocations are rather rare and mostly limited to highly pathogenic strains or predisposing disease states. The small intestinal nutrient rich content at body temperature provides especially ideal growing conditions for microbes, but maintains a rather low numbers of microorganisms. Like the gastric acid proximally, the ileocecal valve, a sphincter muscle located distally from the small intestine, takes part in sealing it off but can surely not block all bacterial transfer (Eizaguirre et al., 1999). Additional factors have to ensure a tightly controlled flora in this compartment and are provided by complex and jointly acting innate immune barrier mechanisms.

1.3 Small intestinal epithelial innate immunity

Since all organisms are constantly subject to threads from viruses over microbes to parasitic worms, they depend on defense mechanisms to limit the presence and growth of infectious agents. Taken together, the protective machinery of an organism is called immune system (Akira et al., 2006). Already the term "immune" (Latin

immunis = free, intact) hints to its ability to limit and eliminate potentially harmful organisms but also foreign substances or endogenous malignant cells. In humans, like in all jawed vertebrates, the immune system has been sectioned into a more ancient or basic part, the innate, and a more "sophisticated" or acquired part, the adaptive immunity. The former recognizes general structures of infectious non-self; the latter is aimed at specific antigens, facilitates the success of vaccines, recognizes also non-infectious structures but can also favor autoimmune disorders, e.g. systemic lupus erythematosus, in case of disturbed self-tolerance (Janeway, Jr., 2001; Janeway, 1989; Ley et al., 2006; Peschel, 2002). One of the biggest variations between innate and adaptive immunity is the reaction time after a potential thread exposure. This is partly due to the mentioned differences in pathogen recognition and more rapid in innate immunity. The innate system relies on non-clonally distributed, genome encoded "pattern recognition receptors" (PRR). Those, unlike adaptive receptors, require no segmental gene rearrangement and monitor microbes by the detection of essential and therefore conserved structures termed "pathogen- associated molecular patterns" (PAMPs) respectively "microbe-associated molecular patterns" (MAMPs) (Didierlaurent et al., 2002). Stimulated PRRs induce a rapid and continuous first line of defense including the production and release of various coordinative signaling molecules (e.g. chemokines), mucins (Cario, 2005; Lievin-Le, V and Servin, 2006) as well as different bactericidal effectors which can additionally bridge towards adaptive immunity (Esche et al., 2005; Yang et al., 2004; Schröder, 1999; Bevins et al., 1999). The immediate and broad reactivity is the main reason for an eminent importance of innate defenses in the GIT (Lievin-Le, V and Servin, 2006a). Prominent intestinal PRR include the constitutively but also inducibly expressed transmembranous surface "Toll like receptors" (TLR´s) found on all GIT cells and the intracellular "Nucleotide-binding oligomerization domain containing molecules" (NODs), which are not exclusively but predominately expressed in small intestinal Paneth cells (Lala et al., 2003). Paneth cells (PCs) are extremely specialized and located at the very bottom of the small intestinal crypts of Lieberkühn. They produce broad- spectrum antimicrobial peptides (AMPs), most abundantly the constitutively expressed α-defensins human defensin (HD) -5 and

-6 (HD5 und HD6, gene names “defensin, alpha” (DEFA)5 and -6) which are also the most dominant AMPs in the small intestine (Jones and Bevins, 1992; Ouellette, 2004; Wehkamp et al., 2006). Those and other in part broadly and constitutively or inducibly expressed AMPs generate a competent weapon arsenal with activity against bacteria, enveloped viruses, protozoa and fungi. Besides helping to avert invasion they also prevent adherence of both, pathogens and commensal bacteria (Wehkamp et al., 2005a; Otte et al., 2003). AMPs share common characteristic biochemical properties like a low molecular mass, a positive charge and disulfide bonds (Boman, 1995). Their mechanisms of action are yet not completely understood but depend on these biochemical properties, e.g. their cationic character which can, for example, in some cases mediate integration into and disruption of negatively charged microbial membranes (Papo and Shai, 2005). Interestingly, different pathogens could adapt towards AMPs and therefore have the potential to evade elimination through this innate immune function (Koprivnjak and Peschel, 2011). There are different bacterial resistance mechanisms but they are often affecting the membrane composition and therefore the interaction with defensins and other AMPs. One example would be *Staphylococcus aureus*, which exhibits cell wall teichoic and lipoteichoic acids, complex surface polymers, and modified membrane lipids to resist the antimicrobial functions of some AMPs (Fedtke et al., 2004).

Besides HD5 and -6, PCs also store and secrete several other innate antibiotic peptides (e.g. lyozyme, regenerating islet-derived 3 gamma (RegIIIγ) and phospholipase A2 group IIA ($sPLA_2$, also known as PLA2G2A)) (Bevins, 2004; George et al., 2008; Wehkamp et al., 2006). Investigations on their antibacterial activity exposed an effective killing capacity of HD5 against *S. aureus* as well as gram negative bacteria, whereas HD6 so far only exhibited little antibacterial potential *in vitro* (Ericksen et al., 2005). Both seem to also have antiviral activity but studies are still emerging (Klotman and Chang, 2006; Doss et al., 2009; Lehrer et al., 2009).

As described, the different innate epithelial derived factors not only inhibit infections but also limit luminal colonization by commensals (Lievin-Le, V and Servin, 2006; Cario et al., 2002). Disturbances in this host defense system are fatal and favor both, increased susceptibility towards pathogenic threads and a misbalance towards the normal flora (Wehkamp et al., 2005a; Bevins and Salzman, 2011). Such events form the core of our hypothesis for the development of chronic intestinal inflammation and will be discussed in the following paragraphs.

1.3 Inflammatory bowel disease

Inflammatory bowel diseases (IBDs) are characterized by recurring and severe inflammations in the intestinal tract with diarrhea, ulcerations and sometimes multiple fistulas (Podolsky, 2002). Additional characteristics like a distorted epithelial architecture, lymphocyte and plasma cell infiltrate, polymorphonuclear cryptitis, crypt abscesses, and basal lymphoid aggregates are used to distinguish chronic IBD from other non-relapsing intestinal inflammations (Le et al., 1995). The most common forms of IBD: ulcerative colitis (UC) and Crohn's Disease (CD), are furthermore differing in clinical features. Whereas UC is for example typically restricted to the rectum and colon, CD can occur at many sites, predominantly in the small intestinal ileum, the colon, or in both locations. Even though many pathologic features might differ in UC and CD, about 5% of cases, termed as "indeterminate" colitis (IC), cannot be definitely diagnosed (Odze, 2003). Up to today it is well accepted that both, genetic but also environmental factors contribute to the development of IBD (Halfvarson et al., 2006; Schreiber et al., 2005). One well established environmental concept is an influence of intestinal microbes. In contrast to the balanced host/microbe relationship in a healthy gut, intestinal epithelia of IBD patients display mucosal adherent bacteria, activated T-cells, and antibodies aimed towards the flora (Duchmann et al., 1999; Sartor, 2001; MacPherson et al., 1996). These observations challenged the earlier pathogenesis concept which was based on an autoimmune concept (Strober, 2006; Levine and Fiocchi, 2000) and provoked an ongoing discussion over the last years. Researchers and clinicians slowly started to favor barrier defect hypotheses and shifted their

scientific focus towards a major role of impaired epithelial innate immunity (Wehkamp et al., 2008; Stange 2009).

The intracellular PRR NOD2, also known as CARD15, a receptor for murmayldipeptide (MDP), the minimal bioactive peptidoglycan motif common to all bacteria (Girardin, 2003) represents the foremost genetic Crohn's disease susceptibility factor (Hugot et al., 2001; Ogura et al., 2001). As it is predominately found in the antimicrobial producing Paneth cells, its association with the disease was the first major genetic mechanism which underlined the importance of innate defenses and the crucial role of bacteria-host interactions in the disease.

1.4 Crohn's Disease

About 5.6/100.000 residents are newly diagnosed with Crohn's disease every year in Europe (Lakatos, 2006; Shivananda et al., 1996). Besides the occurrence of rather frequent and abstract extraintestinal manifestations such as rheumatic, metabolic, or dermatologic complications, intestinal CD symptoms themselves can be quite heterogeneous (Larsen et al., 2010). The Montreal classification therefore subphenotypes CD according to the affected gastrointestinal location and distinguishes between patients with solely the small intestine (L1) or the colon and/or rectum affected (L2) and those with both, small and large intestine, involved (L3). An additionally affected upper GIT is registered separately to the primary location (+L4). Besides the disease location, the patient's age of onset also plays an important part in subphenotyping. As genetics seem to be more important in early onset cases, studying pediatric presentations of the complex disease helps to determine inherited risks. Nonetheless, factors which specifically underlie the early onset phenotype are yet to be identified (de Ridder et al., 2007; Scherr et al., 2009). Of further importance are the disease progression states or more specific the disease behavior which can range from a relatively weak "inflammatory" (B1), to a more sever stenotic (B2), or a penetrating (B3) course with internal fistulae. Whereas behavioral characteristics might change over time, location maintains a stabile entity throughout the course arguing for

differing, likely genetically determined pathogenesis mechanisms in small intestinal and colonic CD subgroups (Louis et al., 2001; Gasche and Grundtner, 2005; Silverberg et al., 2005). In the last years, our group identified different defects in the intestinal antimicrobial defense of CD patients, which could not only explain the location specificity, but additionally accommodate both, the inherited and the microbial component of the disease (Wehkamp et al., 2008). Little is known about *in vivo* effects of common drugs on the expression of antimicrobial defense molecules and since researchers and clinicians are just beginning to fully understand the complex pathogenesis mechanisms, a causal therapy is yet unavailable (Stange et al., 2006; Travis et al., 2006). Anti-inflammatory medications like corticosteroids, thiopurines, methotrexate or tumor necrosis factor (TNF) -antibodies form the current mainstay in therapy and can dampen disease symptoms which follow an adaptive immune response at the mucosa (Travis et al., 2006). Nonetheless the induction and long term maintenance of remission is still unsatisfactory and will require new and curative therapy approaches that target underlying primary disease factors rather than secondary symptoms (Lemann et al., 2006).

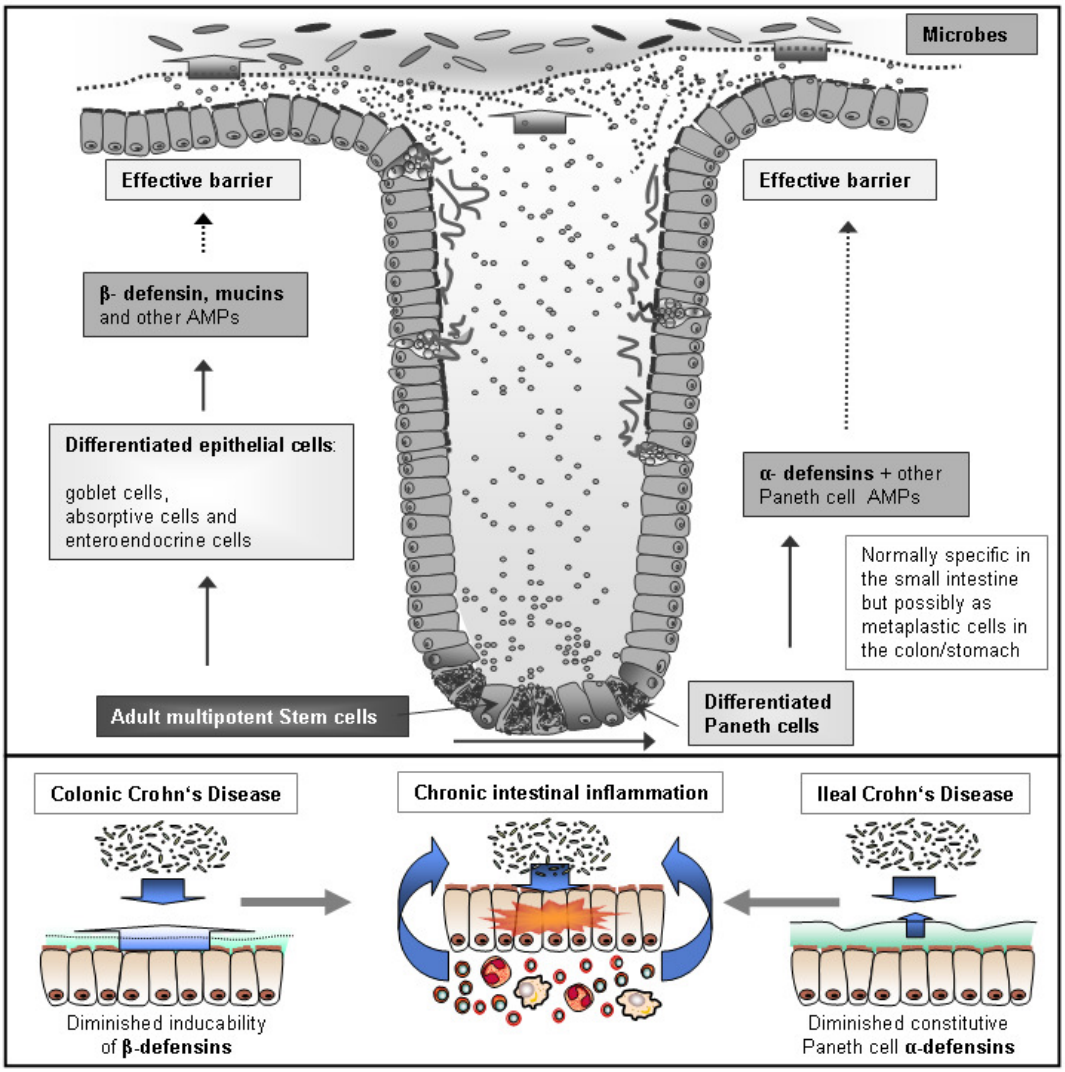

Figure 1.2: **Hypothesis on the pathogenesis of Crohn's Disease:** *Upper Panel*: In a healthy gastrointestinal system, epithelial cells provide a physical shield and produce chemical and biological barriers with essential functions in host defense (e.g. mucus, antimicrobial peptides). Intestinal stem cells give rise to a zone of rapidly cycling transient amplifying precursors which differentiate into the multiple lineages (absorptive enterocytes and secretory cells: goblet, enteroendocrine and Paneth cells). Important barrier factors helping to maintain the mucosal integrity in the small intestine are the Paneth cell α- defensins HD5 and -6. *Lower Panel*: Reduced antimicrobial function as present in Crohn's disease patients diminish the host's capacity to fend off microbial threads and subsequently favor chronic inflammation

1.4.1 Colonic Crohn's Disease

Crohn's disease which is affecting the colon is characterized by an attenuated production of β-defensins e.g. the inducible human-beta-defensin (HBD) -2, also known as defensin, beta 4 (DEFB4) (Kapel et al., 2009; Wehkamp et al., 2003a; Nuding et al., 2007). A constitutively and broadly expressed β -defensin, HBD1 (DEFB1), also shows a reduced expressions in CD patients with colonic involvement. This might partly be explained by a functional promotor single nucleotide polymorphism (SNP) which could be associated with protection from colonic CD in a European cohort (Peyrin-Biroulet et al., 2010). β- defensins share, like already mentioned for most AMPs, common characteristic biochemical properties like a positive charge and disulfide bonds that are important for their antimicrobial function (Boman, 1995). Even though these properties are quite similar between the respective peptides, earlier studies only elucidated a weak antimicrobial function for HBD1. The reason for this became evident in a recent study by Schroeder et al. from our laboratory. They could show that the quite strong antimicrobial mechanism of HBD1 against various commensal bacteria and also a facultative pathogenic fungus depends on its biochemical activation through a reducing environment leading to the opening of disulphide bonds (Schroeder et al., 2011). The published results highlight that previous, often artificial experiment settings, don't have to represent actual circumstances in the organism and might miss important conclusions. It is thus likely, that other defensin functions as well as antimicrobial activities have yet to be elucidated and that the environment plays an important role in that matter.

Different from the constitutively expressed HBD1, HBD2 is induced by different pathogens, which involves the activation of "nuclear factor kappa-light-chain-enhancer of activated B cells" (NF-κB) and activator protein 1 (AP-1) (Ogushi et al., 2001; Wehkamp et al., 2003b). Via the same pathways, probiotic bacteria have also been found to induce HBD2 (Mondel et al., 2008; Schlee et al., 2007; Schlee et al., 2008). A CD specific defect in β- defensin inducibility and/or function might likely explain why probiotic treatment seems to have promising effects in pouchitis and maintenance of

remission in ulcerative colitis, but no benefit in CD (Schultz and Lindstrom, 2008). If and how currently used traditional immunomodulatory therapeutics, such as steroids, affects intestinal β- defensins is so far not clear. Besides bacteria, proinflammatory cytokines, e.g. Interleukin (IL)1β, (O'Neil et al., 1999), IL-23 and IL-17 (Wilson et al., 2007) can induce HBD2 as well as HBD3 (also know as Beta-defensin 103 (DEFB103a)) by similar means. Like the low HBD1 level, diminished HBD2 inducibility can partly be explained by genetics. Different from HBD1 however, this does not include a functional polymorphism in the promotor, but respectively a low copy number of the HBD2 gene (*DEFB4*) (Fellermann et al., 2006). Since data from a New Zealand cohort on an elevated copy numbers in CD irrespectively of location (Bentley et al., 2009) as well as data on unchanged copy numbers (Aldhous et al. 2010) recently challenged the previous findings, further investigations are needed. Such studies as well as additional investigations on colonic antimicrobial activity will help to better understand the pathogenesis and disease etiology in colonic CD.

1.4.2 Ileal Crohn's Disease

In ileal CD, a prominent and primary decrease of the most abundant small intestinal AMPs, the constitutively expressed human defensins -5 and -6 (Jones and Bevins, 1992; Ouellette, 2004; George et al., 2008; Wehkamp et al., 2005b; Wehkamp et al., 2006) endorse our concept of defective antimicrobial defenses as major pathogenesis mechanisms in CD. Both α-defensins are exclusively produced in Paneth cells. Our group initially reported the impairment in patients with ileal involvement (Wehkamp et al., 2004b) and a following study in German and US patient populations confirmed the decrease both for mRNA and protein (Wehkamp et al., 2005b). Expression levels of a total of eight other PC products show no such decrease when compared with controls and the specific defensin reduction stands furthermore independent from inflammation and cannot be observed in colonic CD, UC, or pouchitis, an inflammatory control of non-Crohn's ileitis (Kubler et al., 2009; Wehkamp et al., 2002; Wehkamp et al., 2005b). The decrease is furthermore more pronounced in patients carrying a frameshift mutation in the susceptibility gene *NOD2*. Complementing the finding in

patients, an in vitro model revealed that epithelial cells with the CD specific mutation F3020insC, fail to induce NfκB and the massive AMP response which can be seen in NOD2 wildtype cells (Begue et al., 2006). Besides the reported data on impaired defensin expression, additional reports on a limited function of produced HD5 peptide strengthens the key role of Paneth cell antimicrobials in ileal CD. Since HD5 is released as a propeptide, an enzymatic cleavage is necessary to activate its antimicrobial properties. Pro-HD5 is processed by trypsin in a complex in which chymotrypsinogen is also cleaved for activation in the intestinal lumen. In CD, this complex is irregularly maintained and therefore impairs antimicrobial defenses possibly due to increased luminal levels of proteinase inhibitors (Elphick et al., 2008).

1.5 The major role of Paneth cells in antimicrobial defense

Besides HD5 and -6, Paneth cells produce other broad- spectrum AMPs (as mentioned for example lyozyme, RegIIIγ and sPLA2) which are stored in cytoplasmatic granules (Bevins, 2004; George et al., 2008; Wehkamp et al., 2006). The release of PC antimicrobials follows stimulation of PRRs with bacterial products. Such products include lipopolysaccharide (LPS), which activates TLR4, other bacterial cell wall glycolipids which are recognized independently of TLR4 (Tanabe et al., 2005), MDP, the PAMP recognized by NOD2 (Ayabe et al., 2000) and unmethylated cytidine-phosphate-guanosine (CpG) sensed by TLR9 (Rumio et al., 2004). The functional importance of PC α-defensins, called cryptdins in mice, is illustrated by different models: extensive PC degranulation following TLR9 stimulation protects mice against S. typhimurium (Rumio et al., 2004), mice lacking their cryptdin - activating enzyme are conversely highly susceptible to orally administered pathogens (Wilson et al., 1999), whereas HD5 transgenic mice are again protected against infections (Salzman et al., 2003). The influence of α-defensins on intestinal microbial ecology has additionally been studied. In the latter two complementary models, significant changes in the composition of, but not in the amount of flora hint to an in vivo homeostatic role in the regulation of the commensal makeup (Salzman et al., 2009). The role of PCs in sensing and controlling microbial threads becomes also evident in mice expressing “myeloid differentiation

primary response gene" (MyD88), an important cytoplasmatic TLR signaling compound, in Paneth but not in hematopoietic immune cells. In this setting, the PC-intrinsic MyD88 is sufficient to limit bacterial penetration by triggering a complex antimicrobial program (Vaishnava et al., 2008). In healthy wild type mice, it could furthermore be shown that cryptdins resist proteolysis in vivo, which keeps them at an active and likely flora regulative state in the distal colonic lumen (Mastroianni and Ouellette, 2009). The accumulated data from different mouse models underline a pivotal role of Paneth cells in protecting the organism from food and water-borne pathogens as well as in the host-commensal balance at the small, and potentially even the whole intestinal mucosal barrier. It is imaginable how the diminished expression of both PC α- defensins in ileal CD patients supports increased infection susceptibility and even more a misbalance towards the normal flora. We believe that both favor subsequent inflammations and that their decrease and the underlying defects in ileal CD patients not only provide pathogenesis mechanisms but potential targets for future causal therapy approaches.

1.6 Paneth cell α- defensin impairments and inflammation

Even though multiple studies have reported an inflammation independent decrease of the human defensins 5 and -6, the primary character of their impairment has been under discussion (Simms et al., 2008; Bevins 2009). Both antimicrobials are constitutively expressed and appear to be under the control of different factors, including the disease associated PRR *NOD2*. Nonetheless, even though Paneth cells are located at the very bottom of the crypt right next to the stem cells, and affected by multiple disease linked mutations (Koslowski et al., 2009), one might think that inflammation mediated tissue destruction could still influence their expression levels. Further evidence and investigations will help to evaluate their role and lead to a better understanding of the situation presented in patients. Recently a study by our group on the transcriptional impairment of the Wnt pathway transcription factor TCF7L2 allowed a reconditioned and quite novel view on the primary character of diminished α- defensin levels in ileal CD (Wehkamp et al., 2007). Given that Wnt controls Paneth

cell maturation and intestinal proliferation aside from directly regulating HD5 and -6, the observed link might suggest an involvement of disturbed cell differentiation. The next paragraphs will therefore focus on intestinal epithelial proliferation, Wnt signaling and its influence on Paneth cell maturation and function.

1.7 Intestinal proliferation and Paneth cell differentiation

The human intestinal epithelial lining undergoes cell renewal at an extraordinary rate, outrunning all other tissues of the organism (van-Wetering et al., 1999; Gregorieff and Clevers, 2005). All intestinal cell types descend from multipotent stem cells located at the base of the crypts, right above and/or between the Paneth cells. The adult stem cells self-renew and give rise to daughter cells forming an adjacent zone of rapidly cycling progenitors. Those again increase their pool before differentiating into multiple lineages creating up to 300cells/cryp/day (Winton and Ponder, 1990; Barker et al., 2008). The crypt necks and the villus regions consist of post- mitotic cells and make up the biggest part of the intestinal epithelium (Crosnier et al., 2006). Paneth Cells escape the general upwards flow and migrate downwards to the crypt base where they reside 3–6 weeks (Barker et al., 2008). The maintenance of the proliferating region is in large parts subject to the activity of secreted lipid-modified Wnt glycoproteins (Korinek et al., 1998). A study by Hoffman et al. on physiologic functions of this morphogene used adenoviral expression of the secreted Wnt antagonist Dickkopf-1 (Dkk1) in adult mice. This approach revealed a rapid inhibition of small intestinal and colonic epithelial regeneration, loss of proliferative crypts, and eventual inflammation and architectural degeneration upon inhibition of the pathways activity (Hoffman et al., 2004). The intestine's proliferative capacity is furthermore dependent on Notch signaling. Notch is, in interplay with Wnt, capable of amplifying the progenitor pool while partly inhibiting cell differentiation (Fre et al., 2005; Fre et al., 2009). Both signals, Wnt and Notch are paradoxically also essential for the directed maturation of specific cell types (Jensen et al., 2000; van Es et al., 2005).

1.7.1 The β-catenin dependent Wnt pathway

It is easy to picture that all intestinal proliferation and differentiation events underlie a complicated system of sending, receiving and transferring of various signals. For each of those steps multiple variations are known in the Wnt pathway (Gordon and Nusse, 2006). There is not only a pool of differently operating Wnt molecules, but also various different receptors, as well as intracellular compounds and signaling cascades. A major one, the β-catenin depending cascade (called "canonical Wnt pathway"), depends on activation of Frizzled as well as "low density lipoprotein receptor-related protein" (LRP) 5 or 6 receptors by Wnts and subsequent accumulation of cytoplasmatic β-catenin. The mechanisms by which a Wnt ligand mediates β-catenin stabilization are complex and yet not fully understood. The events during the absence of Wnt are, however, better characterized: β-catenin is associated with a destruction complex containing adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK-3β) and axin amongst others (Fagotto et al., 1999; Aberle et al., 1997; Orford et al., 1997). The destruction complex mediates GSK3β- dependent phosphorylation and subsequent ubiquitination- dependent proteasomal degradation of the protein. An inhibition of the formation and/or activity of this complex are hypothesized upon activation of the canonical pathway. So far it has been shown, that the Wnt ligand- receptor- interaction leads to phosphorylation/activation of dishevelled (Dvl) (Yanagawa et al., 1995; Willert et al., 1997). Dvl interacts and cooperates with different binding partners (e.g. "frequently rearranged in advanced T-cell lymphomas- 1" (Frat-1) and casein kinase I epsilon (CKIε)) and antagonizes GSK3β dependent β-catenin phosphorylation (Hino et al., 2003). Mammals feature 3 known Dvl isoforms which may operate as a network (Lee et al., 2008).

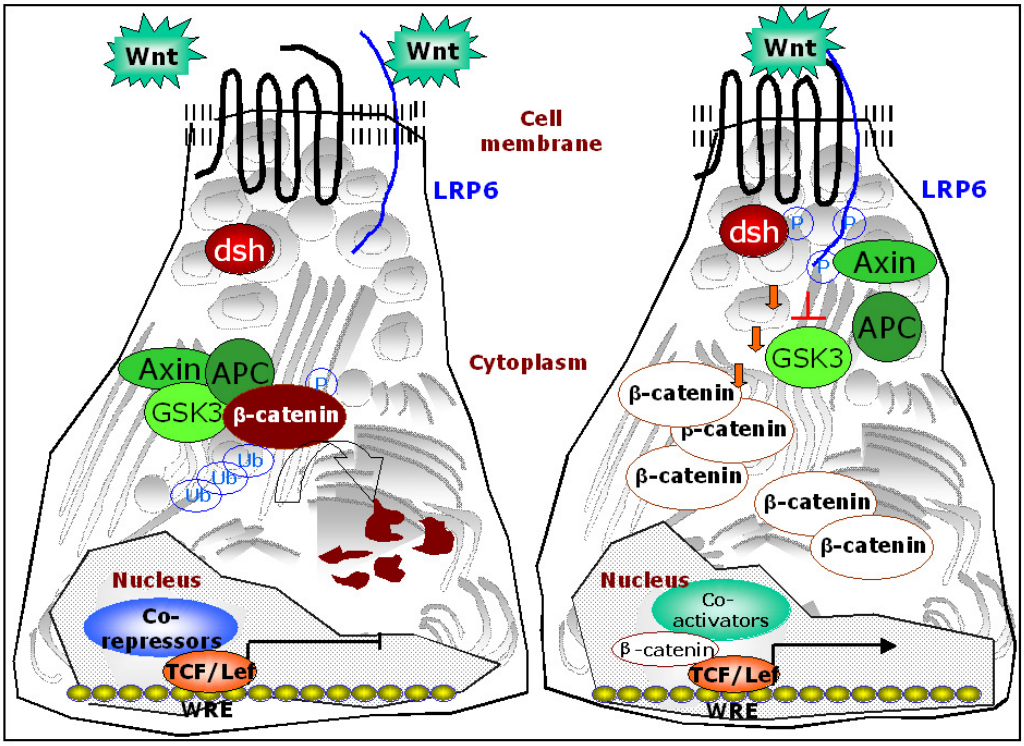

Figure 1.3: **Schematic comparison of the different "canonical Wnt" states**: *Left panel*: In the absence of Wnt β- catenin is associated with a destruction complex and left to phosphorylation- dependent ubiquitynation and proteasomal degradation. *Right panel*: The pathway's activation results from an interaction of Wnt proteins with receptors of the Frizzled and LRP family. This triggers mechanisms which antagonize β- catenin degradation, amongst others, the activation of dishevelled proteins. After stabilization, β- catenin can enter the nucleus and cooperate with transcription factors of the Lef/TCF family. Target genes of the two cooperating transcription factors include HD5 and -6 as well as other critical components of the complex Paneth cell gene program.

Other mechanisms which are promoting β- catenin accumulation are the recruitment and direct inhibition of GSK-3β by activated LRPs (Piao et al., 2008), as well as an additional interaction and consequent degradation between axin and the same receptors (Fagotto et al., 1999; Mao et al., 2001b). In addition there have been various studies done on factors which influence the localization of β-catenin and therefore also the activity of canonical Wnt. Identified in this context have been amongst others, E-cadherin (Muise et al., 2009) and interestingly in a direct manner also Notch (Kwon et al., 2011).

Taken together, cytoplasmatic β-catenin is stabilized by different events reliant on Frizzled and LRP5/6 activation. Accumulated β-catenin can then enter the nucleus where it activates the transcription of target genes in cooperation with transcription factors of the "lymphoid enhancer-binding factor" (Lef)/ "transcription factor (T-cell specific, high-mobility group (HMG)-box)" (TCF) family. TCFs/Lef are sequence-specific DNA binding transcription factors that function in the pathway by recruiting β-catenin to Wnt target gene promotors. In humans the TCF/Lef family has four members: Lef1, TCF7 (also known as TCF1), TCF7- like 1 (TCF7L1, also known as TCF3), and TCF7- like 2 (TCF7L2, also known as TCF4), which exhibit remarkable amino-acid sequence conservation in the HMG DNA-binding domain. Consequently, the proteins share a high affinity for an (A/T) (A/T) CAA (A/T) G DNA motif called Wnt response element (WRE) (Arce et al., 2006). In the absence of the Wnt signal, or respectively nuclear β-catenin, they are thought to acts as repressor by recruiting groucho and other factors to target gene promotors. The group of Hans Clevers could show a critical dependence of the Paneth cell gene program on TCF7L2, a TCF factor which is of particular importance in the embryonic mouse intestinal proliferation (van Es et al., 2005). Analogous, a conditional Frizzled-5 deletion in adult mice precedes conspicuous mispositioning of PCs and abrogated expression of Wnt/TCF7L2 target genes, including cryptdins. (van Es et al., 2005). Those findings are corroborated by studies linking conditional APC loss to enhanced progenitor commitment towards the PC lineage (Andreu et al., 2005) and another study which could illustrate a high PC sensitivity to changes in canonical Wnt dosage (Andreu et al., 2008).

1.7.2 The central role of Wnt signaling in various pathway crosstalks

In the last years, quite a few studies on Paneth Cell lineage allocation emerged, providing an improved insight into the underlying complicated regulatory network. Frequently the identified factors stand in crosstalk with the β-catenin dependent Wnt cascade or are directed by or function upstream of the pathway. The transcription factor forkhead box L1 (Foxl1) is expressed in the gastro- intestinal mesenchyme, has been linked to Paneth cell location (Takano-Maruyama et al., 2006) and is likely

occupying a Wnt upstream position. Knockout of the factor leads to aberrant crypt structure, widely distributed Paneth cells and aberrant expression of *EphrinB*, a classical β-catenin dependent target gene. A partly downstream acting factor of the pathway is the "leucine-rich repeat G protein-coupled receptor" (LGR)5, a probable marker of adult intestinal stem cells (Barker et al., 2007). Deficiency of the Wnt regulated protein results in premature PC differentiation without major effects on other lineages, progenitor proliferation or cell migration in the small intestine. Expression from the LGR5 promoter as well as mRNA level of Wnt target genes are upregulated in LGR5-null mice, pointing to an autoregulatory negative feedback loop (Garcia et al., 2009). In the last year, research on LGR5 and other LGR homologues intensified and helped to improve our understanding of the orphan receptors relationship within the Wnt signaling network. Two groups published almost at the same time, that LGR5 and LGR5 homologues are involved in R-Spondin signaling (Carmon et al. 2011; De Lau et al., 2011). R-spondins (RSPOs), a group of secreted Wnt/β-catenin signaling enhancing proteins, have pleiotropic functions in development and proliferation. LGR4 and 5 can bind R-spondins and also associate with the Frizzled/LRP complex. They function as facultative Wnt receptor components and potentiate Wnt-induced LRP6 posphorylation and thereby downstream β-catenin accumulation. Interestingly another recent study analyzed the role of LGR5 in colon cancer and found that ablation of the receptor leads to an augmentation of invasion and tumorigenicity and an upregulation of Wnt signaling which, similar to the results of Garcia et al., points to a negative autoregulatory feedback mechanism (Walker et al., 2011). The "fibroblast growth factor receptor 3" (FGFR-3) is, like LGR5, expressed in the lower crypt proliferative zone and its knockout is marked by a limited allocation toward the PC lineage and changes is mucosal morphology. Since the total content and nuclear localization of β-catenin is diminished in ko mice, as is expression of canonical Wnt targets, FGFR-3 functions at least partly dependent on β-catenin action (Vidrich et al., 2009). The transcriptional co- repressor "myeloid translocation gene (MTG) related-1" is a further important factor related to crypt cell differentiation (Amann et al., 2005). Mice lacking the co-repressor fail to maintain small intestinal secretory

lineages. A later study reported that MTG family members again provide a link to Wnt, since they associate with and influence TCF and specifically TCF7L2 -dependent transcription (Moore et al., 2008). A role of the Wnt-responsive, Ets-domain transcription factor "SAM pointed domain containing ets transcription factor" (Spdef) in the terminal differentiation of goblet and Paneth cells has recently been shown by Gregorieff et al.. Spdef ko mice manifest impaired maturation of both cell types and an accumulation of immature secretory progenitors (Gregorieff et al., 2009). Conversely, loss of hepatocyte nuclear factor (HNF)-4α, a transcription factor in the adult mouse intestinal epithelium, leads to an increase of goblet cells but an impairment of enterocyte and enteroendocrine cell maturation. Additionally to the effects on linage allocation, increased proliferation and expression of several canonical Wnt target genes, destabilization of cell/cell junctions and heightened intestinal permeability are also evident (Cattin et al., 2009).

Besides holding multiple touch points with other proliferation/differentiation factors and pathways, the canonical Wnt cascade is likely also influenced by and plays a role in infections and inflammatory signaling. The progression of colorectal cancer (CRC) is for example coupled to an augmentation of canonical Wnt and proinflammatory cytokines have been shown to enhance β-catenin/TCF transcriptional activity in this setting. IL1β, thereby produced by tumor associated macrophages promotes phosphorylation (respectively inactivation) of GSK3β and subsequently stabilizes cytoplasmatic β-catenin (Kaler et al., 2009). It has also been shown that IL9 treated or overexpressing mice exhibit upregulated goblet cell associated genes (e.g. Muc2), increased expression of PC specific genes (e.g. PLA2g2a) and PC hyperplasia in small intestinal as well as in colonic mucosa, where this cell type is normally absent (Steenwinckel et al., 2009). Both forms of colonic IBD, UC and colonic CD have been shown to bear increased risk for CRC development in epidemiological studies. An impact of microbiota on colitis associated cancer (CAC) development has also been investigated: in azoxymethane (AOM)-exposed conventional, *Bacterioides vulgatus* mono-associated, and germfree IL10 (-/-) and Myd88(-/-) mice, it could be shown that the

risk for CAC development seems to be TLR/MyD88 dependent and can be altered by manipulation of intestinal microbes (Uronis et al., 2009). Unlike findings in regular intestinal inflammation models, the study demonstrated a direct correlation of colitis severity and CRC and that bacteria- induced inflammation drives invasive carcinoma progression. CRC is not the only GIT malignancy with a connection to Wnt activation and linked to inflammation. Deregulation of NFκB and canonical Wnt is found in the majority (>70%) of gastric cancers (Ooi et al., 2009) and induction of high nuclear β-catenin by *Helicobacter pylori (H. pylori)*, a major cause of gastric malignancies, provides again, a link to bacteria. Besides the hosts genetic predisposition, *H. pylori'*s capacity to inhibit GSK3β activity is likely responsible for the outcome of persistence of intestinal metaplasia (Hung et al., 2009). Intestinal colonization by another Helicobacter species, *Helicobacter hepaticus*, activates NFκB-regulated networks both in the lower bowel but also in liver and promotes hepatic cancer marked by canonical Wnt activation without bacterial translocation or hepatitis induction (Fox et al., 2009). Besides bacteria, there are also other potential pathogens, which can influence epithelial differentiation pathways in the gut. Mice infected with *Trichinella spiralis* (*T. spiralis*), a parasite that drives small intestinal inflammation, show changes in mucosal architecture. *T. spiralis* infection elevates the amount of proliferative cells and Paneth cell numbers at the crypt base leading to an upwards shift of the proliferative zone (Walsh et al., 2009b).

The relationship between infection, inflammatory pathways and canonical Wnt seems to be quite complicated and complex but even more, it is also dual. β- catenin itself functions as a constitutive negative regulator of *in vivo* inflammation. Similar to "nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor" (IkB), the central canonical Wnt pathway component binds NFkB and thereby prevents its activity (Duan et al., 2007). In response to pathogenic (*Salmonella*, *Yersinia*, *Listeria* and *enteropathogenic Escherichia coli* (*EHEC*)) but not non- virulent strain bacteria, the physical interaction between the two signaling components is compromised subsequent to GSK3β dependent β-catenin degradation. Conversely a study in colon

and breast cancer cells could demonstrate that GSK3β inhibition can also alter NFkB activity through β-catenin stabilization, which again links canonical Wnt with inflammatory signaling (Deng et al., 2004). Phosphorylation of GSK3β and β -catenin stabilization seem to provide important control points in inflammatory processes, suggesting that activated β-catenin may be a balancer of bacteria-induced inflammation in general and likely also in the gut. A failure of the system could therefore be fatal, shifting epithelial immune reactions towards a more inflammatory status. In monocytes this is already been shown for GSK3β as the canonical Wnt inhibiting factor has a pivotal role in deciding inflammatory responses after TLR activation in these immune cells (Martin et al., 2005). In addition Manicassamy et al. could show that dendritic cells require β-catenin–dependent signaling to mediate gut tolerance to commensal microbes in mice. Knocking out β-catenin in dendritic cells preceded a reduced frequency of regulatory T cells and anti-inflammatory cytokines, an increase in pro-inflammatory cells and their associated cytokines and an enhanced susceptibility to experimental colitis in this model (Manicassamy et al., 2010). Just recently it could also been demonstrated that levels of Wnt2, a ligand in the canonical cascade, are elevated after bacterial infection. Since an *in vitro* knock down of Wnt2 in epithelial cells enhances bacteria induced IL8, which is conversely less secreted in Wnt2 overexpressing cells, this might be a host strategy to inhibit cell apoptosis and an overshooting inflammatory response during infection (Liu et al., 2011)

Taken together, multiple crosstalks between proliferative, immunological and inflammatory pathways complicate the picture of canonical Wnt signaling function in the human organism and likely especially in the gut.

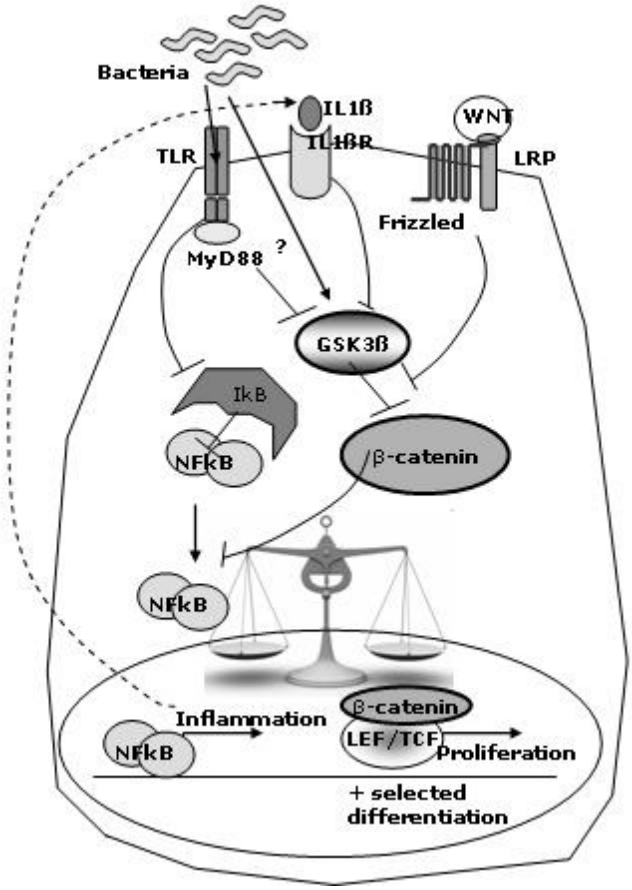

Figure 1.4: **Simplified scheme of multiple crosstalks; Wnt, inflammatory and proliferatory processes need to be tightly balanced:** Dependent on the signaling context and on the strains, bacteria can have the potential to induce canonical Wnt by GSK3β inhibition but also prevent pathway activation by enhanced β-catenin degradation. Inflammatory processes e.g. activation of IL1β signaling seem to induce canonical Wnt thereby favoring overreactive proliferation in certain tissues and malignant conditions. In other tissues, over activated Wnt seems to correlate with increased differentiation and/or decreased proliferation. Inflammatory and Wnt signaling effects are again strongly dependent on the context. B-catenin itself seems to additionally regulate inflammatory outcomes as it partly controls NFκB activity.

An important role of Wnt has already been suggested in other forms of chronic inflammation, for example in rheumatoid arthritis (RA), where bone formation capacity and focal bone erosion repair seems to be impaired despite anti-inflammatory treatment. An upregulation of several Wnt pathway antagonists (e.g. Dkks) in patients

suggests canonical Wnt inhibition as one contributing mechanism and a RA murine model supports a role of deregulated differentiation in inflamed bone (Walsh et al., 2009a). Musculoskeletal complications, e.g. osteoporosis or rheumatic symptoms are frequent and well-recognized in IBD (Rodriguez-Reyna et al., 2009), but if this is due to common underlying pathogenesis mechanisms still remains to be investigated.

1.8 Genetic analysis in humans

Genetics, the science of genes, heredity and variation in organisms, analyses the molecular structure and function of genes as well as their influence within the genome context. Genetic research also deals with patterns of inheritance, with gene distribution as well with variation and changes of genes within populations. As early as 1865, Gregor Mendel established the idea of alleles and dominant or recessive inheritance of traits (Mendel, 1866). It took almost 80 years from then until DNA was isolated (Avery et al., 1944), almost 10 more for James D. Watson and Francis Crick to publish their groundbreaking paper on the molecule's double helix structure (Watson and Crick, 1953) and another 10 years for the genetic code to be cracked with combined efforts of multiple scientist, including Marshall Nirenberg, Heinrich J. Matthaei, Sydney Brenner, Francis Crick, Har Gobind Khorana and Philip Leder in the 1960s (Carlson, 2004). The completion of the Human Genome Project in 2003 (Collins et al., 2003) represents one of the most resent landmarks in genetic research. With this 13 years lasting concerted effort, researchers from all over the world aimed at the identification of the approximated 20.000 – 25.000 human genes and the elucidation of all sequences found in the collective human genome. Within the medical and genetic research community, the project held high expectations to improve our understanding of critical diseases, variances in susceptibility as well as individual differences of patients in the response to therapy. Implementation of genetic criteria to assess specific disease risks or treatment benefits was one of the aspired applications of the gained insights in human genomics (Chiche et al., 2002; Lorentz et al., 2002). Today we know that individual genetics can, in some cases, be used to evaluate disease risks and a need for individualized therapy, but that there are still

various challenges ahead (Offit, 2011). A major task in future genetic research projects will be to further complete the identification of risk markers and potential targets for therapeutic manipulation as well as the incorporation of environmental factors, epigenetic influences, and epistatic effects.

1.8.1 Heredity, genotype, and linkage disequilibrium

The genetic information coded in our genome is transmitted to every next generation in an elaborate molecular process which copies and delivers the DNA to the gametes. Since humans have two sets of chromosomes, two so called alleles exist for every single position of a gene or a specific genetic variant on a chromosome. These alleles collectively form an individual's genotype. During meiosis, the two copies of each of our chromosome can undergo recombination, meaning that DNA segments cross over, possibly relocate and therefore creating new allelic combinations (Youds and Boulton, 2011). Already at the beginning of the last century, Thomas Hunt Morgan described the idea of genetic linkage and hypothesized on the phenomenon of crossing over. Morgan proposed that the amount of recombination between linked genes differs and that crossover frequencies might indicate the distance separating genes on the chromosome. In 1916 he wrote "If we assume that the nearer together the factors lie in the chromosome the less likely is a twist to occur between them, and conversely the farther apart they lie the more likely is a twist to occur between them, we can understand how the linkage is different for different pairs of factors" (Morgan, 1916). Via recombination, a spontaneous mutation can be physically paired with alleles in its vicinity and form a so called haplotype which represents a collectively transmitted combination of alleles. Today we refer to a nonrandom association between alleles at different loci in the genome as linkage disequilibrium (LD). LD is only a measure of association and does not necessarily call for the analyzed alleles to be on the same chromosome. In case of genetic linkage though, an important aspect of LD is that once established, it can again be broken down by recombination. However, in reverence to the distance, or in other words depending on the level of genetic linkage between the two loci, the rate of convergence of LD to zero can be fast or slow. Evolutionary

biologists and human geneticists imply different analysis of LD to estimate recombination rates and to map genes which are associated with quantitative traits (Barton 2011). To indicate the level of linkage disequilibrium two statistical measures are commonly used: D' and r2. D' is a simple test for LD intensity which is the ratio of D (the deviation of the observed frequency of an allelic combination from the expected frequency) to the theoretical possible maximum for the observed frequencies (Lewontin, 1964). The second used measure, r2, represents the square of the correlation coefficient of the allele frequencies and is another normalized LD measure (Hill and Roberts, 1968). D' and r2 have different biological interpretation since D' measures only recombinational history, whereas r2 summarizes recombinational and mutational occurrences and is adjusted for differences in allele frequencies between analyzed loci (Jorde, 2000). The range of values for both measures is: 0 (absolutely no LD) ≤ D' or r2 ≤ 1 (perfect LD). It is furthermore important to acknowledge that the diversity levels within our DNA vary across the human genome in which two distinct forces, differences in mutation rates and the differential impact of natural selection play an important role (Spencer et al., 2006). Natural selection can affect the genetic drift of relevant fitness influencing loci, but also of linked evolutionary neutral sites via selective sweeps (or genetic hitchhiking), and background selection. Genetic hitchhiking thereby occurs due to a strong positive selection, the latter in case of a strong negative effect on the general fitness by a specific locus (Innan and Stephan, 2003). Purging of deleterious variants will result in the occasional removal of linked variants, whereas the specific selection of beneficial alleles might also increase the frequency of surrounding alleles, both producing a decrease in the level of variation around the locus under selection within a population.

1.8.1 Single nucleotide polymorphisms and genetic susceptibility

The dissection of genetic disease causes and the identification and characterization of new candidate genes can be a quite complex task. It can be even more challenging in a disorder like IBD, which is genetically multifactorial and non Mendelian inherited and also characterized by an influence of partly unknown environmental factors (Sartor,

2011; Van Heyningen et al., 2004). Additional characteristics of the disease like the previously described detailed subphenotyping and potentially an involvement of epistatic genetic effects and/or a role of disease modifying genes complicate the picture. The latter as well as general disease causing genes might be unraveled in a correlation of SNPs with clinical indices, disease progression and outcome. Single nucleotide polymorphisms represent the most common form of genetic variants; they affect a single base differing in the DNA sequence between individuals as well as potentially between the chromosomes of one individual. Millions of such variations can be found in the human genome pool, but most have to date no identified effect. In the last years however, more and more associations of common SNPs with different diseases, disease subphenotypes and courses as well as with variability in therapy response, suggest an important role of SNP alleles in an individual's constitution and susceptibility (Janssens et al., 2008; Ginsburg and Willard, 2009). In October 2002 the International HapMap project started to genotype a defined group of individuals from Utah (90 samples from 30 families with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain collection (abbreviated to CEU)) as well as individuals from Nigeria, China, and Japan (The International HapMap Consortium, 2005). The project partners aimed at identifying distinct haplotypes, in this case different collectively transmitted SNPs at different loci, which consequently exhibit a high state of LD. In its first phase, the project successfully genotyped 1.1 million SNPs. This set was expanded during the second phase to include 3.9 million putative polymorphic variants. Even though one third of the tested markers turned out to be non-polymorphic among the CEU population, the remaining confirmed 2.6 million SNPs are estimated to cover 92% of hidden common variants (with a minor allele frequency ≥ 5%) at the threshold of r2 ≥ 0.8 (The International HapMap Consortium, 2007).

Genetic research approaches in humans, which target an involvement of SNPs, follow the "common disorder, common variant" principle. It is based on the idea that genetic variants which influence an at least fairly common disease trait, will, to a reasonable

extent, be common in the population. Genome-wide association studies (GWAS) represent a quite broad SNP genotyping approach (Gibbs and Singleton, 2006). They use high-throughput technologies to determine the distribution of hundreds of thousands of SNPs, which ideally cover all known haplotypes in a population and relate them to clinical conditions and measurable traits.

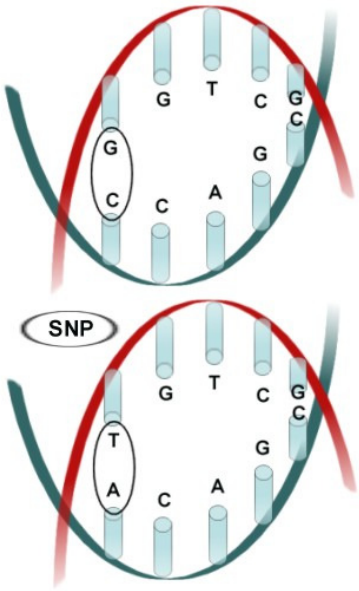

Figure.1.5: example of a SNP: 2 DNA molecules with differing base pairs: The one above G/C and at the same position in the other strands T/A. SNPs can lie outside or inside gene coding regions. The latter can furthermore be synonymous (non coding, silent) or non-synonymous (coding, resulting in a different amino acid after translation). A SNP can also constitute a deletion or insertion of a single nucleotide and, when present within a coding region, can lead to a frame shift in the transcription of the affected gene.

Different from GWAS, which have an extraordinary divers but hypothesis- free character, the candidate gene approach requires not only a general hypothesis on involved pathways, signaling networks or mechanisms in a disease, but also a clear idea about affected key factors. A limiting issue in both approaches is the size of the analyzed population. Is it too small, weaker effects (low odds ratio) or variants with a high effect but a low minor allele frequency in the study population might be lost due to inefficient statistical power (Ioannidis et al., 2010). On the other hand, too much statistical power might lead to an overwhelming amount of significant but biologically

rather unimportant associations. It is furthermore extremely hard to discriminate between disease causing genes and disease course modifying genes in an analysis which solely relies on genetic association. Investigations on epistatic effects are also rather impossible. A GWAS analysis would have to include all so far known risk genes and SNPs even though they might not achieve statistical significance in the tested population. It would further require the use of adequate algorithms to unravel potential interactive effects between certain alleles. Such an approach would not only imply an adequate test population, but also a respective amount of manpower, platform technologies as well as biostatistics and genetic epidemiology software to integrate the results (Cantor et al., 2010). In addition to the ambitious genome wide studies, investigations on the distribution of SNPs are also performed to strengthen an already developed hypothesis in a proof of principle manner. In this case, it is important to select the most plausible candidate genes and the most promising SNP variants for an analysis in an excellently phenotyped cohort. A large part of the herein presented work deals with such genotyping experiments, subphenotyping and data analysis in the context of small intestinal CD to support our hypothesis of defective antimicrobial defenses and an involvement of impaired Wnt signaling in the disorder.

1.9 Hypothesis and aims of the work

Crohn's Disease, a chronic inflammatory disorder of primarily the lower gastrointestinal tract, affects about 100-150 out of 100.000 German citizens. Since there is so far no cure, patients are often required to deal with lifetime therapy and monitoring. Different from earlier investigations following the lead-off autoimmune pathogenesis concept, a significant number of more recent studies shifted their focus on primary epithelial barrier defects. In the subgroup of small intestinal CD, our group has reported various data on a prominent role of Paneth cell α-defensin (HD5 and -6) deficiencies as underlying disease mechanisms. These defensins are critical innate antimicrobial peptides which avert pathogenic attacks and balance commensal bacteria at the intestinal epithelium. Our group has furthermore reported a connection between their decrease and impairments in the canonical Wnt pathway transcription

factor TCF7L2 (Wehkamp et al., 2007). The Wnt pathway has major functions in development and tissue homeostasis. In the intestine it controls the proliferative potential of epithelia and the maturation and maintenance of Paneth cells and also the transcription of HD5 and -6. Aberration in the tightly controlled Wnt program might therefore have a fatal potential in biasing mucosal barrier effectiveness on multiple levels. If our hypothesis proofs valid, a major mechanistic role of the pathway might be based on genetic aberrations in key factors of canonical Wnt signaling transduction.

In particular the following issues are addressed

I A potential role of TCF7L2 promotor variants in ileal CD

Our group has evidence for a potential association of *TCF7L2* promotor SNP variants with ileal CD in a small IBD cohort from Vienna (Kübler, I, Dissertation 2007). The factor was previously shown to be impaired in its mRNA expression and thereby directs diminished antimicrobial activity in the mucosa of ileal CD patients (Wehkamp et al., 2007). The association was not evident compared to controls, but a statistically significant weak difference in allele frequencies was found in comparison with solely colonic CD. We assumed, that a significant association could be found in a statistically more powerful setting and therefore planned to analyze the variant's distribution in 2 additional IBD cohorts and an increased control population for the previously tested set. We furthermore hypothesized that other genetic variants might be present in the *TCF7L2* coding and splice regions with a potential influence on the genes function and/or expression. A genetic association would not only support the factors important function in patients and argue for a primary role of Paneth cell antimicrobial defects, but also provide a potential marker for hypothetical future gene diagnostic approaches

II A potential influence of upstream Wnt factors in ileal CD

We concluded that, if the role of Wnt is as critical for the disease as we assume other components of the pathway could be affected in patients. Factors with a decisive position in canonical Wnt signaling transduction, are the "low density lipoprotein

receptor-related protein" (LRP) 5 and 6 (Liu et al., 2003; Semenov et al., 2001; Mao et al., 2001a). The Wnt co- receptors are, together with receptors of the Frizzled family, essential for cytoplasmatic stabilization of β- catenin, the central step in the canonical cascade. Interestingly, even though LRP5 and 6 are widely co- expressed, the two factors seem to have also somewhat distinct functions (Holmen et al., 2002). Genetic variances in the essential co- receptors have been associated with bone mass density (BMD) (Sims et al., 2008) and bone disorders (Levasseur et al., 2005; van Meurs et al., 2008). Osteopenia and osteoporosis are frequent manifestations in IBD patients. Even though they are thought to be developed secondary to gut inflammation and/or certain medications (Bernstein, 2006), disease-inherent factors also appear to confer a major risk irrespectively of anti-inflammatory treatment (Frei et al., 2006). LRP6 mutations and or genetic variants have additionally been implied in late-onset Alzheimer's disease (De Ferrari et al., 2007), macular degeneration (Haines et al., 2006) and cardiovascular diseases (Sarzani et al., 2009; Mani et al., 2007) but studies on an involvement in IBD have not yet been conducted. Both Wnt co- receptors seem to underlie genetic variation in the general population as manifested in BMD variability and Alzheimer's' disease susceptibility. Because of this and due to their central role in canonical Wnt, we decided to focus on the two factors. Functional variants in the receptors and/or impairment on the transcriptional level could be involved in defective Paneth cell function in ileal CD. Due to its already investigated role in intestinal tissue (Hoffman et al., 2004) and because a functional Wnt affecting mutation could already be described (De Ferrari et al., 2007), coding variants in *LRP6* were prioritized.

III The effect of standard treatment on intestinal antimicrobial peptides and their dependence on inflammation in active Crohn's disease

Besides investigating disease underlying mechanisms, we also planned to systematically assess the expression of various important intestinal AMPs in affected CD tissue and relate the findings to the degree of inflammation. The aim was thereby to confirm an inflammation independent regulation for some and an inflammation dependent regulation of others, and furthermore define the impact of standard

medications on their expression. So far, probiotic bacteria are the first therapeutic agents used in IBD of which we know they can influence AMP production via pathways involving NFκB and AP1. Nonetheless, therapeutic strategies which broadly suppress inflammation and adaptive immunity might also affect relevant pathway components in the epithelium and subsequently influence mucosal innate immunity in some way.

IV General Discussion and Outlook

Finally, the results will be integrated into the network of current knowledge about CD pathogenesis and heritability. Besides emphasizing the new and important aspects of gained insights, the implications for future research and for clinical practice will be explored.

2. MATERIAL and METHODS

2.1 Patient samples

All patients and healthy controls included in the present studies gave their written and informed consent after the study purpose, sample procedure, and potential adjunctive risks were clarified. All studies were approved by the ethics committees of the respective institutions: the Medical University Vienna, Austria, the University Hospital Tübingen, Germany, the University of Leuven, Belgium and the Oxford Radcliffe Hospital Trust. Subgrouping of included patients was done according to provided phenotype data which was based on clinical, radiological, endoscopic and histopathological diagnoses at the respective IBD centers.

2.1.1 Samples for mRNA expression analysis

All tissue samples used for mRNA analysis were part of the Stuttgart cohort which was continuously collected since 2001 at the Robert-Bosch Hospital, Stuttgart, Germany. Included patients and healthy controls were endoscoped because of diagnostic reasons. Additional biopsies for study purposes were collected from the ileum, cecum, sigma and the rectum and immediately shock frozen and stored in liquid nitrogen. In line with the Montreal classification three CD subgroups were defined to accommodate the different disease locations: ileal disease only (L1), colonic disease only (L2) and ileocolonic disease (L3).

2.1.2 Samples used in genetic analysis

For the sequencing of *TCF7L2* exonic and intron boundary regions we used genomic DNA isolated from blood samples which were collected from individuals included in the Stuttgart biopsy cohort. We randomly selected 10 healthy controls and 25 CD patients who were classified as L1. To analysis the distribution of genetic variants, we evaluated 3 Caucasian DNA cohorts (Oxford, Vienna and Leuven) of CD and UC patients

as well as healthy unrelated controls. DNA was isolated at the respective centers from whole blood via standard procedures and provided as either dried or liquid samples. Phenotyping data which was collected on site was used to classify and group the included individuals in this study. To exclude an effect due to differences between the groups in gender, CD patients were accordingly sub grouped. Additional points of interest were the behavior as well as the onset of the disease. Finally we analyzed patients with an additional involvement of the upper gastrointestinal tract (L4).

2.2 Reagents, chemicals, standards

NEEO ultra quality agarose, chloroform, EDTA, ethanol, HCl, isopropyl alcohol, Tris, sucrose, dimethylformamide, Diethylpyrocarbonate (DEPC) and X-Gal were sourced from Roth (CARL ROTH GMBH + CO. KG, Schoemperlenstr. 1-5, 76185 Karlsruhe, Germany). Ampicillin, ethidium bromide and Luria-Agar for plates as well as Luria Broth medium for cultivation in falcons were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Riedstraße 2, 89555 Steinheim, Germany). Bromophenol blue, xylene cyanol as well the Li Chrosolv water utilized in sequencing reactions was ordered from Merck (Merck KgaA, Frankfurter Str. 250, 64293 Darmstadt, Germany). The for RNA isolations required TRIzol™ reagent, as well as the for gel electrophoresis of polymerase chain reaction (PCR) products used 100kb ladder originated from Invitrogen (Invitrogen, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008, USA). The different media were manufactured with deionised water.

2.3 Kits and enzymes

<u>Sequencing and Genotyping</u>
QIAamp DNA Blood Mini Kit (Quiagen, Quiagen GmbH Germany, Quiagen Straße 1, 40724 Hilden, Germany)
BigDye® Terminator v3.1 Cycle Sequencing kit (Applied, Applied Biosystems, Life Technologies Corporation, 5791 Van Allen Way, CA 92008, USA)
i**PLEX™ Gold assay** for SNP Genotyping (Sequenom, Sequenom Industrial Genomics, 3595 John Hopkins Ct., San Diego, CA 92121, USA)

mRNA Quantification
AMV Reverse Transcription System Kit (Promega, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA)
TOPO TA Cloning® Kit (Invitrogen)
QIAprep spin Miniprep kit (Quiagen GmbH Germany)
EcoRI time saving high fidelity restriction enzyme and buffers (NEB, New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA)
LightCycler® 480 SYBR Green I Master (Roche, Roche Diagnostics Deutschland GmbH, Roche Applied Science, Sandhofer Str. 116, 68305 Mannheim, Germany)

General
HotStarTaq® PCR Kit (Quiagen GmbH Germany)

2.4 Buffers and Media

50x TAE Buffer
used in the preparation of electrophoresis gels and during electrophoresis in chambers

242g Tris
57ml glacial acetic acid
100ml 0.5M EDTA (pH 8.0)
add 1L aqua dest.

6x sample buffer for gel electrophoresis

40g sucrose
0.25g bromophenol blue or xylene cyanol
add 50ml Millipore H2O

LB-Medium for Agar plates
used for plating of bacteria and selection of colonies with the desired plasmids

40 g Lysogeny (also known as Luria) agar
add 1L Millipore H2O
autoclaving and subsequent cooling down to 60°C
1ml ampicillin solution (100mg ampicillin/1ml Millipore H2O)
before utilization for plating: treating of the hardened LB with 200µl X-Gal solution (40mg/ml dimethylformamide) for blue white selection

LB-Medium
20g Lysogeny (Luria) Broth
add 500ml Millipore H2O
0,5ml ampicillin solution

2.5 Primer and Plasmids

Listed are solely primers and plasmids which have been used directly in the presented work. Others found in the listed publications regard the contribution of other authors as stated for each manuscript.

2.5.1 mRNA expression analysis

Primer

Primer for the construction of Plasmid standards and real-time PCRs were designed using freely available online software (Primer3, Steve Rozen and Helen Skaletsky.) and are listed in the following table:

Product	Sequence forward Primer (5' – 3')	Sequence reverse Primer (5' – 3')
HBD-1	TTGTCTGAGATGGCCTCAGGTGGT AAC	ATACTTCAAAAGCAATTTTCCTTTAT
HD 5	GCCATCCTTGCTGCCATTC	AGATTTCACACACCCCGGAGA
HD 6	CCT CAC CAT CCT CAC TGC TGT TC	CCA TGA CAG TGC AGG TCC CAT A
LL- 37	TCGGATGCTAACCTCTACCG	GGGTCACTGTCCCCATACAC
LRP6	TGCCATTGCCATAGATTAC	CCATTGAGCCTTGTCACTTC
B-actin	GCCAACCGCGAGAAGATGA	CATCACGATGCCAGTGGTA

Table 2.1: Primer for the analysis of transcription level of specific products

Plasmids

All plasmids that were used as standards for the calculation of mRNA copy numbers were constructed as described under 2.7.1.3. using the pCR® 2.1-TOPO® vector and PCR products which were constructed with the above listed primer.

2.5.2 Sequencing of TCF7L2 exonic regions

All primers used in the construction of PCR products for sequencing as well as Primers used in the sequencing PCRs were designed using freely available online software (Primer3) and are, together with their locations in the *TCF7L2* gene region, listed in the appendix.

2.5.3 SNP Genotyping

All primers were designed as subsequently described under 2.7.2.4. and are listed in the appendix.

2.6 Further consumables and laboratory equipment

2.6.1 Equipment

Sequencing and Genotyping

Sequencer: ABI Prism™ 310 Sequencer (Applied Biosystems)
MassARRAY® system: SpectroCHIP® arrays, MassARRAY® Nanodispenser and Compact Analyzer (Sequenom, Sequenom Industrial Genomics, 3595 John Hopkins Ct., San Diego, CA 92121, USA)
PCR Thermal Cyclers for 384well format: Veriti 384well cycler (Applied Biosystems)

mRNA Quantification

LightCycler® 480 Real-Time PCR-System (Roche, Roche Diagnostics Deutschland GmbH Roche Applied Science, Sandhofer Str. 116, 68305 Mannheim, Germany)

General
Non listed freezers/fridges, pipettes, incubators, pipetboys, shakers or other non listed equipment were conform to laboratory standards.

Centrifuges: Centrifuge 5415D (Eppendorf), CL- GS6R Beckman Coulter Centrifuge (Beckmann, Beckman Coulter, Inc., 250 S. Kraemer Boulevard, Brea, CA 92822-8000, USA)
PCR Thermal Cycler for tubes or 96 well format: PTC-225 Peltier Thermal Cycler and Gradient cycler (MJ Research now Bio-Rad, Bio-Rad Laboratories, Inc. 1000 Alfred Nobel Drive, Hercules, CA 94547, USA)
Spectrophotometers: NanoDropTM 2000 (NanoDrop, NanoDrop products, 3411 Silverside Rd, Bancroft Building, Wilmington, DE 19810, USA); Bio Photometer (Eppendorf, Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany)
Electrophoresis chamber: "Gelelektrophoresekammer" B1A (Peqlab, PEQLAB Biotechnologie GMBH, Carl-Thiersch-Str. 2b, 91052 Erlangen, Germany)
UV table and camera for the documentation of electrophoresis gels (Cybertech, Berlin, Germany)
8-channel pipettes (Eppendorf)
Liquid Nitrogen tank (Messer, Messer Industriegase GmbH, Messer-Platz 1, 65812 Bad Soden, Germany)

2.6.2 Plastic material and other consumables

Non listed reaction and falcon tubes, Agar plates, 96- and 384well plates and corresponding adhesive films as well as combitips for pipetting were conform to standard laboratory equipment

Tubes: Genetic analyzer tubes and septa (Applied), Cryotubes (Nunc/Thermo Electron LED GmbH, Robert-Bosch-Straße 1, 63505 Langenselbold, Germany), RNAse free reaction tubes (Roth),
96 well plates: LightCycler® 480 Multiwell Plates 96 (Roche, Mannheim, Germany), ultrafiltration NucleoFast® 96 PCR manifolds (Macherey-Nagel, MACHEREY-NAGEL GmbH & Co. KG, Neumann Neander Str. 6-8, 52313 Düren, Germany)
Pipette tips: safe seal tips (Biozym, Biozym Scientific GmbH, Steinbrinksweg 27, 31840 Hessisch Oldendorf, Germany)

2.7 Methods

2.7.1 Molecular biological methods

2.7.1.1 RNA Isolation and cDNA synthesis

All RNA handling steps were carried out using RNAse/DNAse free materials. RNA was isolated using the principle of acidic phenol/chloroform partitioning of RNA into an aqueous supernatant. Frozen biopsies were pestled in liquid nitrogen and lysed with 100µl *TRIzol™* reagent. TRIzol™ contains guanidinium isothiocyanate as a powerful protein denaturant for the inactivation of RNases and RNA was isolated according to the manufacturers protocol (Invitrogen). Briefly after incubation (10min RT), 200 µl of chloroform were added to the tissue - TRIzol™ suspension. After another incubation step (3 min RT) and centrifugation (at 4°C and 11,6rpm) 3 phases could be defined: a colorless aqueous top RNA phase, a middle, grey interphase containing DNA, and a red, organic, phenol-chloroform bottom phase containing proteins and lipids. The uppermost RNA containing phase was transferred into a Dolphin- Eppendorf tube. RNA was precipitated using 99.7% isopropyl alcohol, washed with 75% ethanol, dried, dissolved in 25µl 0,1% DEPC aqua dest., and stored at –80°C. The concentration of RNA was determined using a *nanodrop* spectrophotometer (PeqLab) and the quality was

evaluated by a Technician using the *Agilent RNA 600 nano kit* and the *Agilent 2100 bioanalyzer* (Agilent) according to the manual. Subsequently 1µg of total RNA was transcribed into cDNA with oligo (dT) primers and *AMV- reverse transcriptase* (RT) according to the manufacturer's protocol (Promega). Briefly 1µg RNA was diluted to achieve a volume of 9.75µl and incubated at 70°C for 10min to linearize potential double strand structures. After adding 10.75µl of a mix containing reaction agents according to the manual, the reaction was cycled at 42°C for 15min and then 95°C for 5min in a thermocycler. Finally nuclease free water was added to achieve an end volume of 100µl which was stored at -20°C.

2.7.1.2 DNA Isolation from whole blood

DNA from patients and controls recruited in Stuttgart was isolated by a Technician using a *DNA Blood purification kit* (Quiagen) according to the manufacturer's protocol.

2.7.1.3 Construction of plasmid standards for absolute mRNA quantification of specific products

PCRs with specific primers for desired products were performed with highly specific *HotStarTaq DNA Polymerase* (Quiagen) according to the manufacturer's protocol. Products were validated via gel electrophoresis and purified using the *QIAquick PCR Purification* kit (Quiagen). Concentrations were determined using a *nanodrop* spectrophotometer (PeqLab). To ensure intact poly-A-sequences needed for insertion of the products into a pCR® 2.1-TOPO® vector, cloning of purified DNA was performed immediately after. Cloning of the PCR products and chemical transformation of competent TOP10 *Echerichia coli (E. coli)* (F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) was performed with material from the *TOPO TA Cloning®* Kit (Invitrogen) following the manual. 25 µl and 50 µl each of the transformation reaction were plated on LB agar plates containing 50µl/mg ampicillin and 40µg/ml X-Gal in dimethylformamide to screen for clones harbouring the desired plasmid and incubated at 37°C overnight. White colonies were selected and grown in 5ml LB medium

(50µg/ml ampicillin) in a 37°C shaker overnight. The *QIAprep spin Miniprep* kit (Qiagen) was employed for isolation of plasmid DNA according to the manufacturer's instructions. Briefly, bacteria were lysed and DNA was denatured in an alkaline buffer. Neutralization of the lysate resulted in the exclusive solution of DNA in the liquid phase, which could be separated by centrifugation. Afterwards, columns were used for purification and retained plasmid DNA was eluted in nuclease free H_2O. DNA concentrations were determined using a *nanodrop* spectrophotometer (NanoDrop)

2.7.1.4 Restriction enzyme digestion

EcoRI time saving high fidelity restriction enzyme (NEB) was used to characterize plasmids. The inserted DNA sequence was excisioned at the specific recognition sites present in the vector. 1 restriction enzyme unit/µg DNA and adequate buffers were used according to the manufacturer´s instructions. The reaction volume was adjusted to 20µl with H_2O followed by incubation for 5min at 37°C. Afterwards, the product was applied on a 1% agarose in 1xTAE gel to evaluate the resulting fragment sizes.

2.7.1.5 Sequencing of plasmid standards

The insert of constructed and harvested plasmids were controlled by Dye- termination sequencing based on the chain-terminator method according to Sanger (Sanger et al., 1977) using the ABI Prism™ 310 Sequencer (Applied biosystems) and the *BigDye® Terminator v3.1 Cycle Sequencing* kit (Applied biosystems). The specifically labeled chain terminator ddNTPs permitted sequencing in a single reaction. The modified DNA polymerase enzyme and special labeling dyes of the kit minimized effects due to chain terminator incorporation differences which would result in unequal peak heights and shapes after capillary electrophoresis. Sequence reactions were prepared and conducted according to the manufacturer's instructions (Applied biosystems). DNA was precipitated, washed and dissolved in nuclease free water (Merck) before capillary electrophoresis. Chromatograms and sequences were evaluated using ABI Prism™ Sequence Analyzer software programs (Applied biosystems) and checked against National Center for Biotechnology Information (NCBI) reference sequences.

2.7.1.6 Real-time RT PCR

Quantitative real-time PCR is based on the same principles as the common PCR. The different application, quantification of specific DNA molecules, is achieved by adding fluorescent dyes like *SybrGreen* that can intercalate with double-stranded DNA, as well as the measurement of a melting curve. The interaction of *SybrGreen* leads to an increase of emitted fluorescence from the dye and can therefore be used to detect newly synthesized amplicon DNA after each cycle of the PCR. The additional determination of a melting gradient for the assembled products allows testing for specificity. Standard DNA plasmid samples with the appropriate insert were used in each run for absolute quantification, as they allowed the generation of a calibration curve. Their concentration was determined on a *nanodrop* spectrophotometer (NanoDrop). The relevant Plasmid standards were diluted from 1ng in a 1:10 manner till a concentration of 10^{-7}ng was achieved. For the quantitative PCR of standards and samples, we used the LightCycler® 480 Real-Time PCR-System (Roche) and the 480 SYBR Green I Master according to the manuals. Each qRT-PCR program started with a denaturation step at 96°C for 2 seconds followed by 40 amplification cycles (temperature transition rate of 20°C/second). Annealing was performed at 66°C (HD5, HD6), 62°C (Lysozyme, LRP6), 60°C (β-actin), and 54°C (LL37) for 8sec and followed by an elongation phase for 10sec at 72°C. After the run, a melting curve analysis was performed as well as a quantification of the products via LightCycler® Software 3.5.

2.7.2 Genetic analysis

2.7.2.1 *NOD2* genotyping

Genotyping of the herein used samples for the common disease associated *NOD2* variants (SNP8, SNP12, and SNP13) has already been established within the methods framework of the working group. It was performed by a Technician using TaqMan technology (Applied Biosystems, Foster City, California, USA) according to the manuals and as described previously (Wehkamp et al., 2004b).

2.7.2.2 Sequencing of *TCF7L2* exonic and intron boundary regions

During the previous sequencing of the *TCF7L2* putative promoter region (2.1 kb upstream) in randomly selected healthy controls (n=10) and patients with ileal CD (n=10) variants which seemed to be more frequent in patients were identified (Kübler, I, Dissertation 2007). In addition, we herein carried out a sequence analysis of known *TCF7L2* exons including ~100 bp intron boundaries in 10 randomly chosen controls (6 identical to promoter analysis) as well as 25 patients with ileal CD (7 identical to the previous promoter analysis). Primers to create PCR products as well as additional primers for sequencing reactions were designed using ENSG00000148737 of the Ensemble genome browser database and Primer3Plus (Untergasser et al., 2007). Specific products were amplified via PCR and validated on 1% agarose gels. When reaction products appeared specific and clean, the DNA was further processed using vacuum-driven purification on ultrafiltration NucleoFast® 96 PCR manifolds (Macherey-Nagel), recovered in 25 µl nuclease free H_2O and, together with the designed primers shipped on dry ice for further analysis. Sequencing was performed according to standard procedures (Sanger principle) by the group of Matthias Chamaillard in France.

2.7.2.3 Candidate Gene and SNP selection

Focusing primarily on and selecting LRP6 as an ileal CD candidate gene was based on literature research. Nonetheless we also decided to include LRP5 variants. We prioritized LRP6 SNPs of the coding gene regions (synonymous as well as non-synonymous variants), included additional SNPs from the literature and from intron boundary regions as well as putative regulatory regions after screening the single nucleotide polymorphism database (SNPdb) within the NCBI. Finally Tag SNPs for LRP6 were included to cover potentially missed disease associated variants in intron-, 3'- or 5'-regions. The selection of Tag SNPs was carried out in a "pairwise method" using the Tag SNP picker function of the International HapMap Project homepage. The online available application is based on "Tagger" software (developed by Paul de Bakker at the Center for Human Genetic Research of Massachusetts General Hospital and Harvard Medical School, and the Broad Institute, USA). To include promoter variants

and other SNPs up- or downstream of LRP6, we zoomed out of the gene region (10% ~ 7.3 kbp) before running the application. Additional search criteria included a minor allele frequency (MAF) cut off of 0.15 and an r^2 cut off of 0.8. Since our study cohorts include solely Caucasians, Tag selection was based on genotyping data from the HapMap CEU population. Potentially functional variants in the LRP6 coding regions were prioritized and therefore the main focus for assay designs. The inclusion criteria for other selected SNPs in genotyping assays were based on compatibility with the prioritized SNPs.

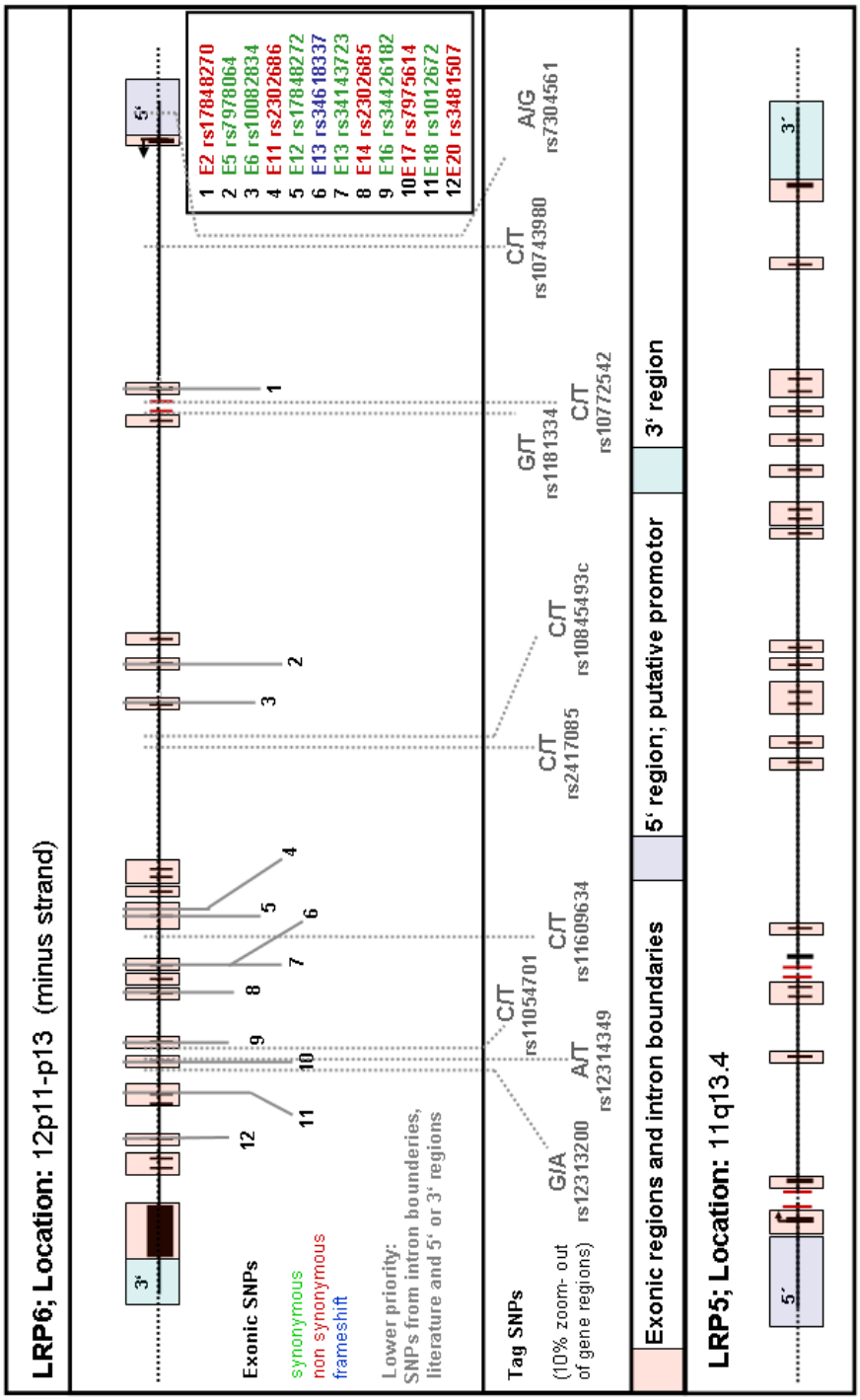

Figure 2.1: Target gene cards and SNP selection: LRP6 was prioritized but also variants of LRP5 were chosen for a high-throughput SNP analysis candidate gene approach. 3 Assays were designed to cover all exonic LRP6 SNPs. Inclusion of LRP6 Tag SNPs and other variants as well as LRP5 SNPs in exonic regions and intron boundaries, putative promoter regions and the untranslated 3' ends which might influence mRNA stability was based on compatibility and primer design using specialized MassARRAY® Assay Design Software.

2.7.2.4 SNP Genotyping

Genotyping was performed using a matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) based mass spectrometry (MS) of allele specific primer extension products using a MassARRAY® Compact System from Sequenom (San Diego, USA) and

materials from the Sequenom iPLEX® Gold Kit and were used according to the manufacturer's protocol (Sequenom). The MALDI-TOF type of analysis measures the time of flight of ionized molecules to determine their masses. It was first used in protein analytics (Hillenkamp et al., 1991; Karas and Hillenkamp, 1988) but has been adopted to detect SNP variants in DNA fragments (Ross et al., 1998; Griffin et al., 1997). Primers for 3 multiplex assays were designed using the reference sequence NT 030059 (for TCF7L2), NT 009714 (LRP6), and NT 033903 (for LRP5) as listed in the SNPdb (NCBI) as well as specialized MassARRAY® Assay Design Software. To avoid extreme differences in the GC content, all primer sequences were adjusted with a preceding 5' ACG TTG GAT G 3' sequence. All oligonucleotides were purified by HPLC, desalted and scaled at 0,04µmol by the manufacturer (Metabion, Martinsried). First, respective fragments for our analysis (~ 100bp including the SNP) were assembled via a Multiplex-PCR using the *HotStarTaq ™ Master Mix* Kit (Qiagen) and verified via gel electrophoresis. The reaction was subsequently subject to a shrimp alkaline phosphatase (SAP) clean- up procedure (Amersham) to remove remaining, unincorporated dNTPs. Samples were treated with the SAP reaction mix and placed in a thermocycler. The dephosphorylation of dNTPs was performed at the ideal SAP working temperature (37°C) and subsequent incubation at 85°C inactivated the enzyme. After SAP clean- up, a Primer extension (PEX) reaction was performed. This linear PCR reaction with specifically designed nucleotides in a single termination mix (MassEXTEND®) allows the subsequent detection of sequence differences according to differences in mass at the single nucleotide level. The assay design furthermore supports universal reaction conditions for all included Primers.

a) Reagents	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	~10ng (dried)
H_2O (nuclease free)	Add 5 µl

b) Reagents	Amount per well
H_2O (nuclease free)	1.53 µl
SAP	0.3 µl
10xbuffer	0.17 µl

Table 2.2: Preparation of fragments for subsequent PEX reactions: a) Multiplex PCR reagents: DNA samples are previously dried in 384 well formats, Primers were previously mixed. b) SAP cleanup step reagents.

Reagents	Amount per well
iplex buffer	0.2 µl
iplex termination mix	0.2 µl
Pex Primer (500.0 µM)	0.0112 µl each
iplex enzyme	0,041 µl
H_2O (nuclease free)	Add 2 µl

Table 2.3: Multiplex PEX PCR reagents. Primers for each multiplex assay were previously mixed.

a) Multiplex- PCR		
Initiation	95° C	15 min
Denaturation	95° C	30 sec
Annealing	56° C	60 sec
Elongation	72° C	60 sec
Cycles	45	
Final elongation	72° C	10 min
Cooling	12° C	*„for ever"*
b) SAP clean- up step		
1. Incubation	37° C	20 min
2. Incubation	85° C	10 min
Cooling	12° C	*„for ever"*
c) PEX reaction		
Initiation	95° C	4 min
Denaturation	99° C	30 sec
Annealing	52° C	30 sec
Elongation	72° C	30 sec
Cycles	56	
Final elongation	72° C	2 min
Cooling	12° C	*„for ever"*

Table 2.4: Thermocycler conditions for the different reactions in the preparing of the samples for MALDI-TOF MS based genotyping using the MassARRAY® Compact System from Sequenom (San Diego, USA)

A PEX reaction cleanup step (exchange of cations) with 6 mg of Resin was used to optimize mass spectrometry analysis of the extended reaction products. Resin is added directly to the primer extension reaction products to remove salts such as Na^+, K^+, and Mg^{2+} ions, which, if not removed, can result in high mass spectra background noise. After adding 15µl nuclease free water, the plates were closed and shaken for 20 min

before they were subjected to a 20 min centrifugation step at 1300 rpm. Samples were then spotted on SpectroCHIP® arrays utilizing the MassARRAY® Nanodispenser and analyzed. The SpectroCHIP® arrays were therefore placed into the MALDI-TOF mass spectrometer which determined the mass correlating genotype in real time. Results were automatically loaded in a specialized database which allowed subsequent analysis with Sequenom software.

2.7.3 Computer analysis and statistics

In silico screen of the 10 kb *TCF7L2* upstream region was performed using "Promoter 2.0: for the recognition of PolII promoter sequences." TESS (Transcription Element Search System) database software allowed assessing of potential binding sites for certain transcription factors in the candidate sequence. Polymorphisms were tested for Hardy–Weinberg equilibrium using freely available Finetti specialized software (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) using log likelihood ratio chi square test in the three cohorts. To test for associations within our genetic analysis (comparing IBD subgroups versus controls) this software was used to calculate odds ratios (OR), Confidence Intervals (CI) and to perform Pearson's goodness-of-fit chi-square tests. Differences in genotype frequencies were subject to both *t* tests and Armitage´s trend tests. Linkage disequilibria and haplotype blocks were calculated with Haploview. To avoid statistical biasing due to multiple testing between different subgroups in the LRP6 analysis, we calculated Bonferroni adjusted p-values for the tests performed with the early onset ileal CD group in the overall sample set. To exclude a coincidental association of the *TCF7L2* SNP rs3814570, the significance of p-values set as < 0.05 was verified using Benjamini- Hochberg correction in the overall group. MRNA levels of analyzed factors were normalized to β- actin and evaluated by GraphPad Prism Ver. 4.0. To analyze expression differences and especially the effect of the LRP6 Ile1062Val variant between the groups we performed non- parametric statistical *U* tests (Wilcoxon- Mann- Whitney- test). Finally Spearmen rank (Spearman r) analysis was used to test for correlation.

2.8 Overview

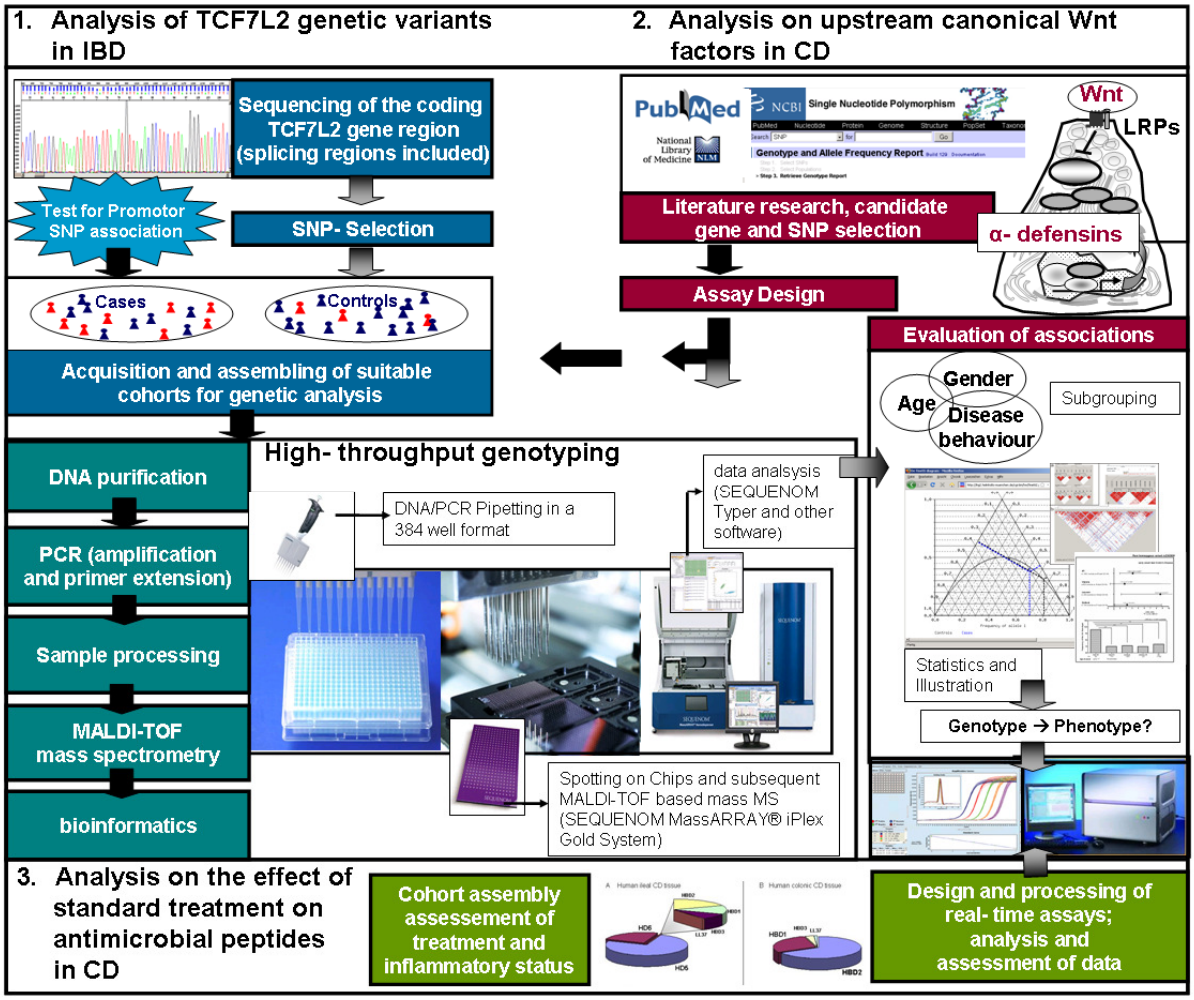

Figure 2.2: An overview on the approaches in this work: 1. TCF7L2 has previously been identified as a candidate gene in ileal CD (Wehkamp et al., 2007). We planned to test if selected *TCF7L2* promotor variants exhibit an association with the disorder in a large cohort. Additionally we sequenced coding and splicing regions of the gene in a subset of ileal CD patients and healthy controls to identify further potentially disease associated variants. High- throughput genotyping for the selected single nucleotide polymorphisms (SNPs) was performed using a MALDI-TOF MS based SEQUENOM MassArray® System. 2. After literature search *LRP6* was prioritized as another candidate gene for a high- throughput SNP analysis approach and further investigations in ileal CD patients. 3. To elucidate potential effects of standard CD treatment and inflammation severity on mucosal innate defenses, we determined expression levels of major ileal and colonic antimicrobial peptides in a respective patient cohort.

3. RESULTS and DISCUSSION

3.1 Genetic variants of Wnt transcription factor *TCF-4* (*TCF7L2*) putative promotor region are associated with small intestinal Crohn's Disease

Maureen J. Koslowski, Irmgard Kübler, Mathias Chamaillard, Elke Schaeffeler, Walter Reinisch, Guoxing Wang, Julia Beisner, Alexander Teml, Laurent Peyrin-Biroulet, Stefan Winter, Klaus R. Herrlinger, Paul Rutgeerts, Séverine Vermeire, Rachel Cooney, Klaus Fellermann, Derek Jewell, Charles L. Bevins, Matthias Schwab, Eduard F. Stange and Jan Wehkamp; Open access via: www.plosone.org

Synopsis

Background

Diminished level of the two ileal antimicrobials HD5 and -6 could previously be linked with reduced expression of the Wnt transcription factor TCF7L2 and subsequently reduced binding of nuclear protein extracts from patient biopsies to WRE in the HDs promotor regions. The identification of TCF7L2 as a new factor in the pathogenesis of ileal CD might provide a quite general mechanism for a deficit in Paneth cell function. Since the transcription factor is directly involved in regulating *HD5* and *HD6* promotor activity, its diminished levels, which are likely based on changes in its own transcriptional regulation, could account for decreased antimicrobial function in a majority of patients. Associations of genetic variations in *TCF7L2* with other diseases exist, but so far never included chronic inflammatory disorders. An association of two non-coding SNPs in *TCF7L2* has been observed with diabetes mellitus (Grant et al., 2006) and, in another study, an association between deletions and insertions of adenines in the coding region was reported in patients with colorectal cancer (Duval et a., 1999). Identifying the factor as a susceptibility gene could provide further evidence for the importance of both, the Wnt pathway and antimicrobial defense at the small intestinal barrier in small intestinal CD. *TCF7L2* putative promotor candidate SNPs for a high throughput screening were identified previously by sequencing of respective gene regions (Kübler, I, Dissertation 2007). The minor T- allele of a selected variant (rs3814570) located in a highly likely regulatory region, exhibited strong and sole linkage disequilibria (LD) with two other putative promotor SNPs, as well as a higher

frequency in patients and was therefore chosen for further analysis in different cohorts from Vienna, Leuven and Oxford (altogether more than 3000 DNA samples)

Main results

In a hypothesis driven candidate gene approach, the frequency distributions of a sequence polymorphism in the *TCF7L2 (TCF4)* promoter were investigated in healthy controls and IBD patients. The rare T allele of rs3814570 was most prevalent in patients with an ileal involvement and no association with either isolated colonic CD or UC was found. Even though the association with small intestinal involvement in CD was evident in all three cohorts, a statistical significance was only achieved in the Oxford sample set and the overall analysis including all controls and patients. In contrast to UC patients who exhibited frequencies similar to controls, CD patients in total exhibited a weak association for the variant (OR 1.18, 95% CI 1.01 to1.39, p = 0.04358). Consistent with our initial hypothesis, investigation of the different CD subgroups revealed an association of the minor variant (T) with ileal CD specifically (OR 1.23, 95% CI 1.07 to 1.41, p = 0.00371), but not colonic CD. Detailed phenotyping of the patients allowed further specifying the variant's potential influence in the disorder. The distribution seemed to be independent of gender but slightly more pronounced in patients with a late disease onset and most prevalent in patients with stricturing behavior or an additional involvement of the upper gastrointestinal tract. Excluding NOD2 mutated individuals revealed unchanged rs3814570 frequencies leaving the genetic variant at a likely independent stand from the pattern recognition receptor. In general, carriers of the minor (homozygous or heterozygous) allele exhibit a higher (~ 28% increased) risk of developing CD with small intestinal involvement. To get an idea about a potential dose effect of the variant, homozygous carriers were analyzed separately. Due to their low numbers and the relatively moderate effect of the gene variant, the about 41% increased risk only achieved statistical significance when all samples were combined.

Variability in allele frequencies of the control groups from Oxford and the two cohorts from the European mainland might be explained by population differences as a

consequence of heterogenic ethnical history. For variants in *NOD2* an even greater heterogeneity among Europeans has been reported (Medici et al., 2006). In a letter regarding the frequency variability of "*disks large homolog 5"* (*DLG5*) polymorphisms, a gene for which an association with IBD has been reported, Tenesa et al. caution against pooling data from different populations, because true but different effects in the cohorts might be concealed (Tenesa et al., 2006). The overall analysis of controls versus ileal CD showed a marginally lower effect when compared with the individual results of the Vienna and Oxford cohorts, so this might play a role in our analysis. However, differences in overall allele frequencies in the control cohorts were not statistically different.

The identification of a genetic association in addition to the previously described functional involvement of TCF7L2 in ileal CD (Wehkamp et al., 2007) strengthens our concept of a primary role of Paneth cells and their antimicrobial function in the disease. Most importantly, our concept provides an alternative therapeutic target to the current merely anti-inflammatory and immune suppressive approaches in Crohn's Disease therapy.

Own contribution

My contributions to this work were in conceiving the study design for the sequencing of the *TCF7L2* gene region, as well as the preparation of PCR products, the assay design and high- throughput genotyping of Oxford, Leuven and additional blood donor samples for rs3814570 using a MALDI-TOF MS based system from Sequenom. Additionally I performed the genetic analysis including the subgrouping of samples according to specific phenotypes, computational and data analysis including the *in silico* screen of the 10kb TCF7L2 upstream region, the calculation of odds ratios, CIs and p- values and finally the preparation of all graphs and writing of the manuscript.

Other contributions

I. Kübler designed and performed sequencing assays for the TCF7L2 promotor region and the genotyping assays for the measurement of SNP distributions using a System from Bruker and genotyped parts of the samples from Vienna. M. Chamaillard and L. Peyrin-Biroulet sequenced PCR products of TCF7L2 exonic regions and were involved in discussion. E. Schaeffeler provided support in the design of, as well as in performing and evaluating of genotyping assays. S. Winter provided support in statistical analysis. W. Reinisch, A. Teml, P. Rutgeerts, S. Vermeire, R. Cooney, D. Jewell provided the DNA as well as organized phenotyping information for the population cohorts and were involved in discussion. G. Wang was involved in conceiving the study. K. Fellermann designed the *NOD2* genotyping assays. J. Beisner, R. Herrlinger and K. Fellermann were involved in discussion. E.F. Stange was involved in discussion and writing of the manuscript. J. Wehkamp was decisively involved in conceiving the study, discussion and writing of the manuscript.

OPEN ACCESS Freely available online

PLoS one

Genetic Variants of Wnt Transcription Factor *TCF-4* (*TCF7L2*) Putative Promoter Region Are Associated with Small Intestinal Crohn's Disease

Maureen J. Koslowski[1], Irmgard Kübler[1,3], Mathias Chamaillard[2], Elke Schaeffeler[1], Walter Reinisch[4], Guoxing Wang[1], Julia Beisner[1], Alexander Teml[1], Laurent Peyrin-Biroulet[5], Stefan Winter[1,6], Klaus R. Herrlinger[3], Paul Rutgeerts[7], Séverine Vermeire[7], Rachel Cooney[8], Klaus Fellermann[3], Derek Jewell[8], Charles L. Bevins[9], Matthias Schwab[1,10], Eduard F. Stange[3], Jan Wehkamp[1,3]*

1 Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, University of Tübingen, Stuttgart, Germany, **2** Inserm, U801, Institut Pasteure Lille, Université Lille 2, Lille, France, **3** Department of Internal Medicine I, Robert Bosch Hospital, Stuttgart, Germany, **4** Department of Internal Medicine IV, Medical University of Vienna, Vienna, Austria, **5** Service d'Hépato-gastro-entérologie, CHU Nancy-Brabois allée du Morvan, Vandceuvre-lès-Nancy, France, **6** Division of Mathematics, Institute of Stochastics and Applications, University of Stuttgart, Stuttgart, Germany, **7** Division of Gastroenterology, University of Leuven Hospitals, Leuven, Belgium, **8** Medical Science Division, John Radcliffe Hospital, Oxford, Oxford, United Kingdom, **9** Department of Microbiology and Immunology, School of Medicine, University of California Davis, Davis, California, United States of America, **10** Department of Clinical Pharmacology, University of Tübingen, Tübingen, Germany

Abstract

Reduced expression of Paneth cell antimicrobial α-defensins, human defensin (HD)-5 and -6, characterizes Crohn's disease (CD) of the ileum. TCF-4 (also named TCF7L2), a Wnt signalling pathway transcription factor, orchestrates Paneth cell differentiation, directly regulates the expression of HD-5 and -6, and was previously associated with the decrease of these antimicrobial peptides in a subset of ileal CD. To investigate a potential genetic association of *TCF-4* with ileal CD, we sequenced 2.1 kb of the 5′ flanking region of *TCF-4* in a small group of ileal CD patients and controls (n = 10 each). We identified eight single nucleotide polymorphisms (SNPs), of which three (rs3814570, rs10885394, rs10885395) were in linkage disequilibrium and found more frequently in patients; one (rs3814570) was thereby located in a predicted regulatory region. We carried out high-throughput analysis of this SNP in three cohorts of inflammatory bowel disease (IBD) patients and controls. Overall 1399 healthy individuals, 785 ulcerative colitis (UC) patients, 225 CD patients with colonic disease only and 784 CD patients with ileal involvement were used to determine frequency distributions. We found an association of rs3814570 with ileal CD but neither with colonic CD or UC, in a combined analysis (allele positivity: OR 1.27, 95% CI 1.07 to 1.52, p = 0.00737), which was the strongest in ileal CD patients with stricturing behaviour (allele frequency: OR 1.32, 95% CI 1.08 to1.62, p = 0.00686) or an additional involvement of the upper GIT (allele frequency: OR 1.38, 95% CI 1.03 to1.84, p = 0.02882). The newly identified genetic association of *TCF-4* with ileal CD provides evidence that the decrease in Paneth cell α-defensins is a primary factor in disease pathogenesis.

Citation: Koslowski MJ, Kübler I, Chamaillard M, Schaeffeler E, Reinisch W, et al. (2009) Genetic Variants of Wnt Transcription Factor *TCF-4* (*TCF7L2*) Putative Promoter Region Are Associated with Small Intestinal Crohn's Disease. PLoS ONE 4(2): e4496. doi:10.1371/journal.pone.0004496

Editor: Amanda Ewart Toland, Ohio State University Medical Center, United States of America

Received June 19, 2008; **Accepted** December 5, 2008; **Published** February 16, 2009

Funding: JW is an Emmy Noether scholar of Deutsche Forschungsgemeinschaft (DFG) who supported this work (WE 436/1-1). This work was also supported by the Robert Bosch Foundation (Stuttgart, Germany), the Ernst Jung Foundation (Hamburg, Germany), the National Institute of Health (AI32738 and AI50843), as well as the DCCV (German patient organization of Crohn's disease and ulcerative colitis). S. V and P. R. are supported by the Funds for Scientific Research (FWO), Flandres, Belgium.

Competing Interests: MJK, JB, EFS and JW have a pending patent application regarding TCF-4 SNP detection.

* E-mail: jan.wehkamp@ikp-stuttgart.de

Introduction

Inflammatory bowel disease (IBD), a chronic inflammation of the intestine, is commonly classified into ulcerative colitis (UC) and Crohn's disease (CD) on the basis of clinical features and histopathology [1]. Whereas UC is typically restricted to the colon, CD can occur at many sites, predominantly in the small intestinal ileum, the colon, or in both locations. Emerging details of disease pathogenesis support the current concept that ongoing immune activation in IBD is driven by bacterial microbiota, possibly as a result to an attenuated antimicrobial barrier in genetically predisposed individuals [1–3]. Both UC and CD have a complex polygenic, multifactorial background, with a coincidence of susceptibility genes and environmental factors involved in pathogenesis. It is likely that different genetically affected factors may explain the various clinical patterns of IBD, especially location of disease in CD, which is stable over time [4–6]. Different explanations for disease location, including a central role of small intestinal Paneth cells and other defects in intestinal innate immunity, were the focus of recent discussion [2]. For ileal CD, reduced expression of small intestinal Paneth cell α-defensins HD-5 and -6 (DEFA5 and DEFA6) has been described in several cohorts [7–12]. The defensin deficiency is proposed to attenuate the antibacterial host defense capacity of the intestinal mucosa, and may initiate and/or perpetuate the chronic inflammation characterizing the disease at this site [7–12]. We recently reported

one mechanism to explain, in part, the decrease of these antimicrobial peptides [9,13]: A reduced expression of the Wnt pathway transcription factor TCF-4 (also known as transcription factor 7-like 2), which directly controls Paneth cell defensin expression (HD-5, HD-6, and orthologous mouse cryptdin peptides [9,13]).

Wnt proteins are a family of secreted morphogenes that play an important role in regulating cell fate and differentiation during embryogenesis [14]. The Wnt signalling pathway is induced by binding of Wnt family proteins to cell surface receptors, leading to stabilization of cytoplasmatic β-catenin, translocation of this regulatory protein into the nucleus, formation of a complex with transcription factors of the Tcf/Lef family and subsequently the activation of various target genes [13]. In the small intestine, epithelial cells transit through differentiation steps initiated in progenitor cells, which reside adjacent to Paneth cells at the base of the crypts [15]. Wnt signalling helps to maintain an undifferentiated state of the intestinal stem cells [16,17] and, paradoxically, also regulates positioning, differentiation and maturation of Paneth cells [13,18]. The Paneth cell gene program is critically dependent on TCF-4 [13]. Using a rodent model, we observed that very small changes (a 50% decrease of TCF-4 levels) are sufficient to compromise mouse Paneth cell cryptidin expression as well as its corresponding antimicrobial function against several bacterial species. We also reported that a reduced level of TCF-4 expression and activity was associated with a decrease of Paneth cell α-defensin levels in CD of the small intestine. The decrease of TCF-4 expression was found to be independent of inflammation in the tissue specimens, and also independent of the 1007fsinsC SNP in *NOD2*, a mutation in this pattern recognition receptor which has previously been associated with ileal CD [9]. We hypothesized that decreased TCF-4 expression might be the result of primary genetic variances in *TCF-4*, at least in some patients with ileal CD. Since there was a decrease in *TCF-4* mRNA levels in these studies, an aberration in the promoter region of *TCF-4* could be a possible explanation. Thus, the aim of this study was to sequence the promoter region of the *TCF-4* gene in a group of patients with ileal CD to identify potential polymorphisms and to perform a subsequent association study on candidate genetic variants in well-defined cohorts of patients. We identified a total of 8 SNP variants, of which three (rs3814570, rs10885394, rs10885395) were in linkage disequilibrium and seemed to exhibit a higher frequency in ileal CD patients. One of these SNPs was found to be located in a putative regulatory region. We carried out high- throughput analysis of this SNP in three IBD cohorts from Oxford, Leuven and Vienna [19–21]. Herein we report an association of the SNP rs3814570 with ileal involvement of CD, but not with colonic CD or UC.

Methods

Patients and human material

For genetic analysis, we obtained DNA samples from a patient cohort of Caucasians with Crohn's disease (N = 259) or ulcerative colitis (N = 149) from the University Hospital in Vienna, as well as a control group of unrelated, healthy Caucasian blood donors in Stuttgart (N = 833). For subsequent testing, we obtained DNA samples from Caucasians with Crohn's disease (N = 277), UC (N = 74) and healthy controls (N = 242) from the University of Leuven, Belgium (*3*) as well as an additional third Caucasian cohort from Oxford with DNA of Caucasian healthy individuals (N = 324), UC (N = 562) and CD (N = 473) patients. In line with the Montreal classification (*4*) three subgroups were defined: ileal disease only (L1), colonic disease only (L2) and ileo colonic disease (L3). A total of 1399 randomly recruited healthy control individuals, 785 UC patients, 225 CD (L2) patients with disease limited to the colon and 784 CD patients with ileal involvement (L1+L3) were used to elucidate the frequency distribution of SNPs [19–21]. The numbers of patient subgroups and controls in the different cohorts are shown in Table 1 and detailed statistical analyses are provided in Table 2. To exclude major differences between the groups in age or gender, CD patients as well as controls were sub grouped according to these criteria (Table 3). Additional points of interest were the behaviour as well as the aggressiveness of the disease. We therefore decided to separately test for an association with inflammatory, stricturing and penetrating behaviour as well as an association of the variant with surgery for Crohn's disease. Finally we checked patients with an additional involvement of the upper gastrointestinal tract (L4). The study was approved by the ethics committees of the Medical University Vienna, Austria, the University Hospital Tübingen, Germany, the University of Leuven, Belgium and the Oxford Radcliffe Hospital Trust. All patients gave informed and written consent for their DNA to be analyzed for this study.

Sequencing of *TCF-4* promoter and gene region

To determine possible genetic variants in the *TCF-4* promoter, we sequenced the 2.1 kb upstream region of randomly selected healthy controls (n = 10) and patients with ileal CD (n = 10). In addition, we sequenced the region of the *TCF-4* gene in which functional insertions and deletions have been reported in colonic cancer [22]. Subsequently, a sequence analysis of known *TCF-4* exons was carried out, including ~100 bp intron boundaries, to identify additional potential variants of this gene in these regions. Primers were designed using ENSG00000148737 of the Ensemble genome browser database for the promoter and exon sequencing. Sequencing was performed according to standard procedures and the primers are provided upon request.

TCF-4 genotyping

Leukocyte DNA was isolated by standard procedures (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany) from whole blood samples. Genotyping of the samples from the cohorts from Vienna and Leuven was performed using the matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) based mass spectrometry (MS) of allele specific primer extension products with a system from Bruker (Daltonik,Leipzig, Germany). Presence of *TCF-4* SNPs detected by MALDI-TOF MS was confirmed by TaqMan® analysis and direct sequencing in a subset of samples. MALDI-TOF MS based genotyping of the DNA samples obtained from Oxford was carried out using a MassARRAY® Compact System from Sequenom (San Diego, USA). Primers were designed using reference sequence NT 030059 and will be provided on request.

NOD2 genotype analysis

Genotyping for the common *NOD2* variants (SNP8, SNP12, and SNP13) was performed in the Vienna patient samples using

Table 1. Overview of the origin of samples from IBD patients and healthy controls.

	Controls	UC	CD (L1)	CD (L2)	CD (L3)
Vienna	833*	149	54	55	150
Leuven	242	74	81	45	151
Oxford	324	562	94	125	254

*healthy blood donors from Stuttgart.

doi:10.1371/journal.pone.0004496.t001

Table 2. *TCF-4* (*TCF7L2*) rs3814570 frequency distribution and statistical analysis of combined cohort samples.

All													
	controls	UC	CD (L1)	CD (L3)	CD (L1+L3)	CD (L2)	CD	IBD	controls			controls	
	n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	n(%)	<> CD			<> UC	
									C<>T	CC<>CT+TT	C<>T	CC<>CT+TT	
rs3814570	1399 (100%)	785 (100%)	229 (100%)	555 (100%)	784 (100%)	225 (100%)	1009 (100%)	1794 (100%)	1.16; p = 0.02246	1.18; p = 0.04358	0.98; p = 0.78946	1.01; p = 0.94441	
									Armitage's trend		Armitage's trend		
C/C	797 (56,97%)	446 (56,82%)	113 (49,34%)	287 (51,71%)	400 (51,02%)	133 (59,11%)	533 (52,82)	979 (54,57%)	1.15; p = 0.02852		0.97; p = 0.79641		
C/T	488 (34,88%)	282 (35,92%)	94 (41,05%)	209 (37,66%)	303 (38,65%)	73 (32,44%)	376 (37,27%)	658 (36,68%)	controls		controls		
T/T	114 (8,15%)	57 (7,26%)	22 (9,6%)	59 (10,63%)	81 (10,33%)	19 (8,44%)	100 (9,91%)	157 (8,75%)	<> L1+L3		<> L2		
									C<>T	CC<>CT+TT	C<>T	CC<>CT+TT	
C	2082 (74,41%)	1174 (74,78%)	320 (69,87%)	783 (70,54%)	1103 (70,34%)	339 (75,33%)	1442 (71,45%)	2616 (72,91%)	1.23; p = 0.00371	1.27; p = 0.00737	0.95; p = 0.67656	0.92; p = 0.54665	
T	716 (25,59%)	396 (25,22%)	138 (30,13%)	327 (29,46%)	465 (29,66%)	111 (24,67%)	576 (28,54%)	972 (27,09%)	Armitage's trend		Armitage's trend		
									1.20; p = 0.00528		0.97; p = 0.68946		

C<>T allele frequency difference.
CC<>CT+TT allele positivity; frequent homo vs heterozygous and rare homozygous.
The different distribution of genotypes is demonstrated for each group and subgroup: controls, inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), CD with solely colonic involvement (L2) and CD with solely ileal (L1) as well as ileal and colonic involvement (L3). Differences in genotype distribution compared to controls in general as well as the amount of all carriers (allele positivity) were subject to t- tests in patients with UC, CD and the CD subgroups L2 as well as L1+L3. Finally, results of the Armitage's trend tests for verification of significant associations of the rare T- variant are shown.
doi:10.1371/journal.pone.0004496.t002

TaqMan technology (Applied Biosystems, Foster City, California, USA), as described previously [7].

Computer analysis and statistics

In silico screen of a 10 kb *TCF-4* upstream region was performed using "Promoter 2.0: for the recognition of PolII promoter sequences." TESS (Transcription Element Search System) database software allowed assessing of potential binding sites for certain transcription factors in the candidate sequence. Polymorphisms were tested for Hardy–Weinberg equilibrium using Finetti specialized software (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) using log likelihood ratio chi square test in the three cohorts. For genetic analysis (comparing IBD subgroups versus controls) we used this software to calculate odds ratios, Confidence Intervals (C.I.) and to perform Pearson's goodness-of-fit chi-square tests. Differences in genotype frequencies were subject to both *t* tests and Armitage's trend tests. Values below 0.05 were considered significant. Linkage disequilibrium between *TCF-4* SNPs and haplotype blocks were calculated and identified using Haploview. To exclude a coincidental association of the SNP rs3814570, the significance of p- values<0.05 was verified using Benjamini- Hochberg correction in the overall group.

Results

SNP selection and haplotypes

To investigate potential genetic linkage of *TCF-4* to ileal CD, we screened for SNPs by sequencing 2.1 kb of the 5′ flanking region of *TCF-4* in a random group of 10 ileal CD patients and 10 healthy controls. We found eight SNPs in this putative promoter region (Figure 1), of which three (rs3814570, rs10885394, rs10885395) were in linkage disequilibrium (LD) in both the patient and control groups. In the control group, two of ten individuals carried the variants; in patients with ileal CD, six of ten individuals were heterozygous for the SNPs. On the basis of these findings, we studied a well-defined cohort of patients with CD and healthy controls from Vienna, Austria. In both the control and CD groups, we found LD between the 3 SNPs that defined a novel haplotype block (Figure 2a).

An *in silico* promoter and transcription factor binding-site analysis of the sequenced region revealed a potential regulatory region close to the location of rs3814570. Because of (i) the observed decreased expression of *TCF-4* mRNA, (ii) the higher frequency of the promoter variant in patients as well as (iii) the presence of a putative regulatory locus, we tested the hypothesis that rs3814570 exhibits an association with small intestinal involvement of CD. To exclude additional major variants in the gene region and possible LD of the identified promoter SNPs to other potentially functional variants in the *TCF-4* gene, we sequenced known coding exons, with ~100 kb overlapping intron boundaries in 10 randomly chosen controls (6 identical to promoter analysis) as well as 25 patients with ileal CD (7 identical to promoter analysis) (Figure S1). We found ten additional putative SNPs, of which two were in LD, but none exhibited LD with the described promoter SNPs (data not shown). A further search for haplotypes in *TCF-4* was conducted based on published data from the HapMap project (Figure 2b), and no haplotype block including rs3814570 or additional SNPs in the gene region were identified.

A *TCF-4* promoter variant is associated with ileal CD predisposition

Analysis of SNP rs3814570 frequency distribution was carried out in a total of 1399 controls (T allele frequency = 25.59%), 785

Table 3. Patients and controls sub grouped according to age and gender. Shown are percentages of individuals per group as well as the *TCF-4* (*TCF7L2*) rs3814570 T- allele frequency (minor allele frequency MAF). Differences in genotype distribution compared to controls in general as well as the amount of all carriers (allele positivity) and the amount of homozygous carriers were subject to t-tests in patients with ileal CD. Finally, results of the Armitage's trend tests for verification of significant associations of the rare T-variant are shown.

Age and gender										
overall		**controls***	**L1**	**L2**	**L3**	**ileal CD**	**CD**		**controls <> L1+L3**	
Age groups	**A1** (<16 Y)	*2,42%*	5,88%	6,02%	9,76%	8,64%	8,06%	statistics for A3	**C<>T**	**CC<>CT+TT**
	MAF	*34,85%*	26,92%	23,08%	23,58%	24,24%	24,05%		1.37; p = 0.06312	1.32; p = 0.20576
	A2(16–40 y)	58,22%	72,85%	68,06%	80,85%	78,53%	76,22%		Armitage's trend	CC<>TT
	MAF	**25,28%**	31,06%	24,15%	29,61%	**30,00%**	28,85%		1.37; p = 0.07315	2.02; p = 0.04347
	A3(>40 y)	39,35%	21,27%	25,93%	9,39%	12,83%	15,71%	statistics for A2	**C<>T**	**CC<>CT+TT**
	MAF	**25,28%**	27,66%	23,21%	35,29%	**31,63%**	28,57%		1.27; p = 0.00567	1.36; p = 0.00438
gender	**male/M**	58,21%	50,88%	35,11%	40,43%	43,46%	41,59%		**Armitage's trend**	**CC<>TT**
	MAF	25,63%	33,91%	20,89%	28,57%	30,38%	28,59%		1.22; p = 0.00818	1.39; p = 0.08396
	female/V	41,79%	49,12%	64,89%	59,57%	56,54%	58,41%			
	MAF	25,44%	26,13%	26,71%	30,15%	29,14%	28,53%			

MAF = minor allele frequency.
*controls A1 only from Leuven.
doi:10.1371/journal.pone.0004496.t003

UC patients (T allele frequency = 25.22%), 225 CD patients with L2 classification (T allele frequency = 24.67%), and 784 CD patients with ileal involvement (L1+L3) (T allele frequency = 29.66%). In contrast to UC (OR 0.98, 95% CI 0.85 to 1.13, n.s.) which was similar to controls, the CD patients in aggregate exhibited a weak association for the minor variant (T allele positivity: OR 1.18, 95% CI 1.01 to1.39, p = 0.04358) (Table 2). Consistent with our initial hypothesis, investigation of the different CD subgroups revealed an association of the variant (T) with ileal CD (OR 1.23, 95% CI 1.07 to 1.41, p = 0.00371),

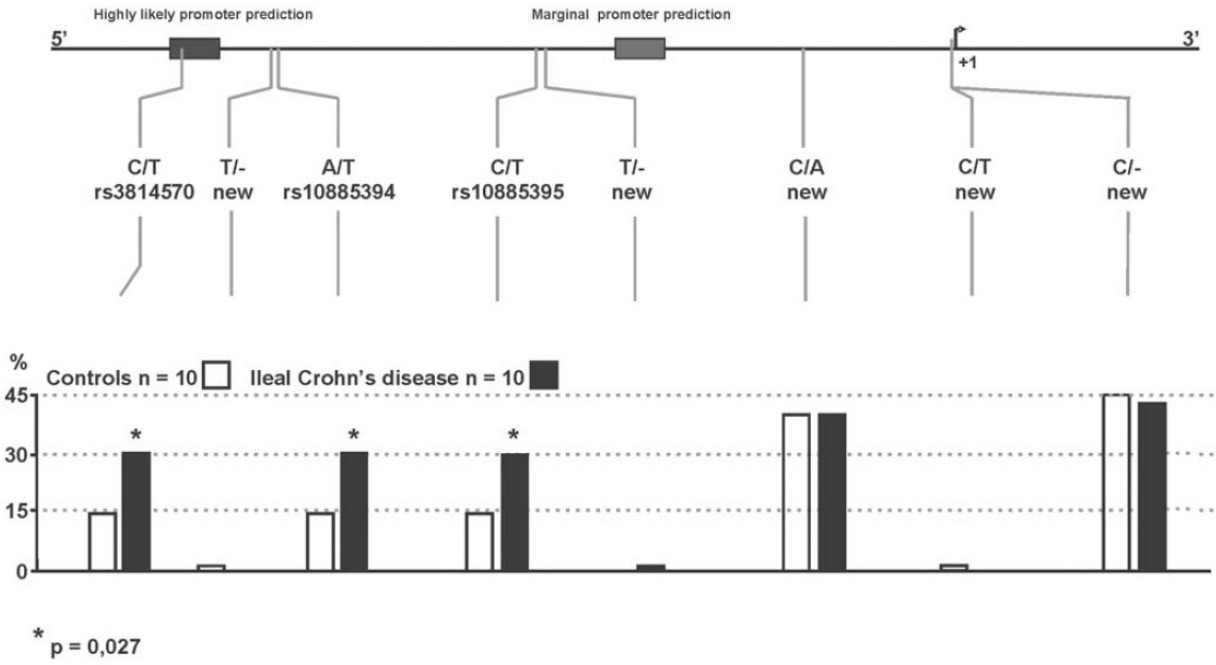

Figure 1. Sequencing of *TCF-4* (*TCF7L2*) 5′upstream putative promoter region. Sequencing of a 2.1 kb upstream region was performed in 10 healthy controls and 10 patients with ileal Crohn's disease. Putative regulatory regions were determined using promoter prediction software. Likely and marginal prediction sites are depicted as red boxes (upper panel). Relative location of identified variants is marked via grey dashes (upper part) and their allele frequency is demonstrated via bars for controls as well as patients (lower part).
doi:10.1371/journal.pone.0004496.g001

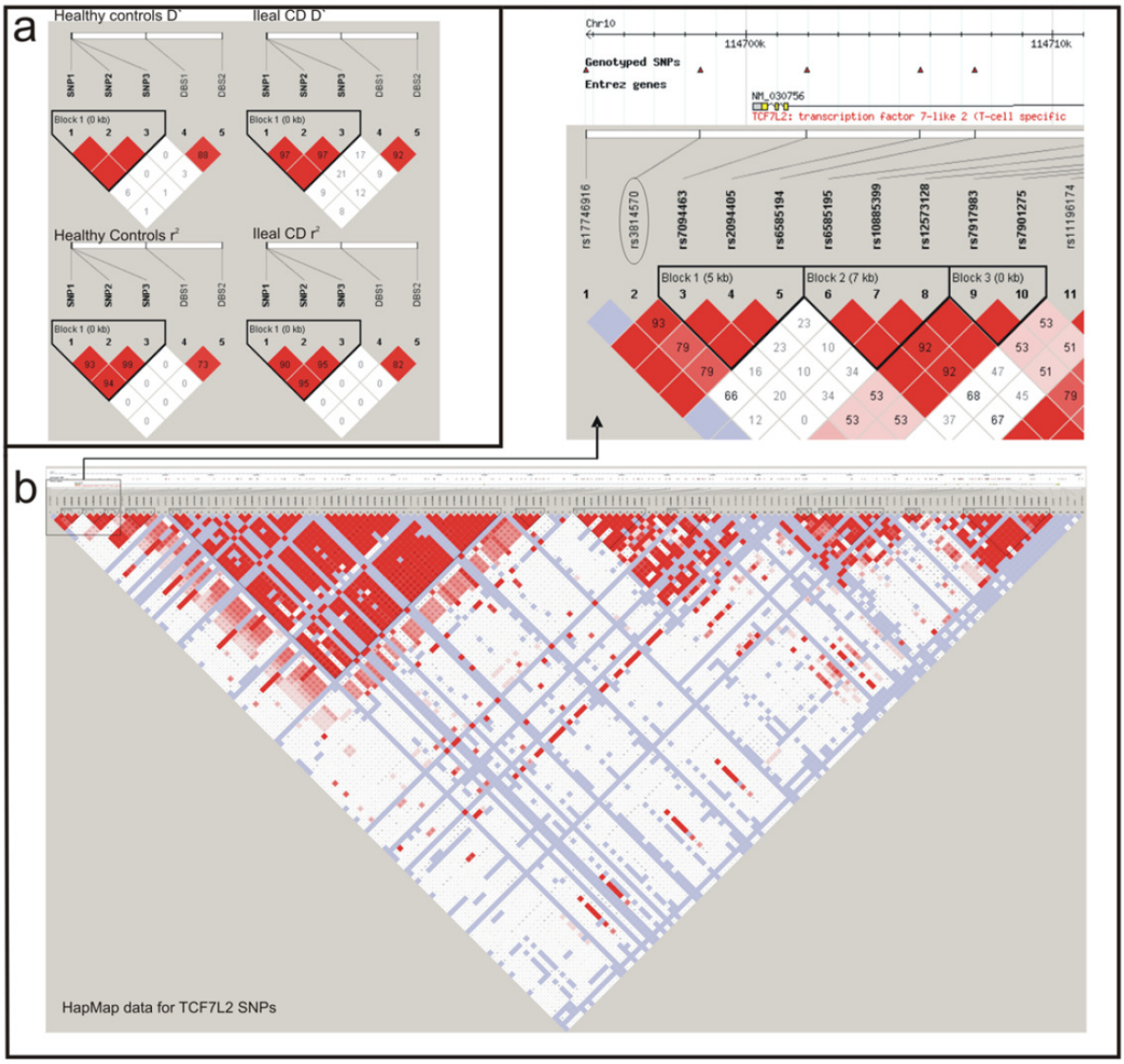

Figure 2. Distribution of haploblocks of *TCF-4* (*TCF7L2*). Both colour schemes (a and b) illustrate the linkage disequilibria. The variants are listed in the upper part of a and b, respectively. Haplotypes for *TCF-4* (*TCF7L2*) rs3814570 (SNP1), rs10885394 (SNP2), rs10885395 (SNP3) and SNPs associated with diabetes in the Vienna cohort are shown in a. A missing number for D′ or r^2 equals 1. Figure 2b: HapMap data based haplotype blocks and linkage disequilibria (LD) for *TCF-4* (*TCF7L2*) polymorphisms. The intensity of red colouring in b is proportional to the extent of D′ or r^2 respectively and a missing number for each of them equals. The observed SNP in the putative promoter region is not part of any significant haplotype block.
doi:10.1371/journal.pone.0004496.g002

but not with colonic CD (OR 0.95, 95% CI 0.76 to 1.20, ns). Testing for allele positivity by analyzing wildtype homozygous individuals (CC) versus all carriers of the minor variant (CT+TT), revealed the effect more clearly comparing healthy controls versus ileal CD (OR 1.27, 95% CI 1.07–1.52, p = 0.007372). Odds ratios and confidence intervals of the group analysis' in the respective cohorts as well as the combined analysis of all genotyping results are shown in Figure 3.

Since there were differences in allele frequencies between the cohorts (Tables S1, S2, and S3), we tested if those apparent frequency differences were statistically significant. In general the Oxford cohort exhibited a lower T allele frequency in controls (23.30%) compared to Leuven (26.65%) as well as to Vienna (26.17%) The same was true for CD patients (T allele frequency in Oxford: 27.38%, Leuven: 30.14% and Vienna: 28.96%), but could partly be explained by the different percentage of colonic CD patients in the groups. For CD with ileal involvement only, the frequency distributions in the cohorts were more similar (T allele frequency in Oxford: 28.30%, Leuven: 30.82% and Vienna: 30.64%) and not significantly different. Even though we found a possible change in frequency distribution between the Oxford control group with both the Leuven (allele frequency: OR 1.20,

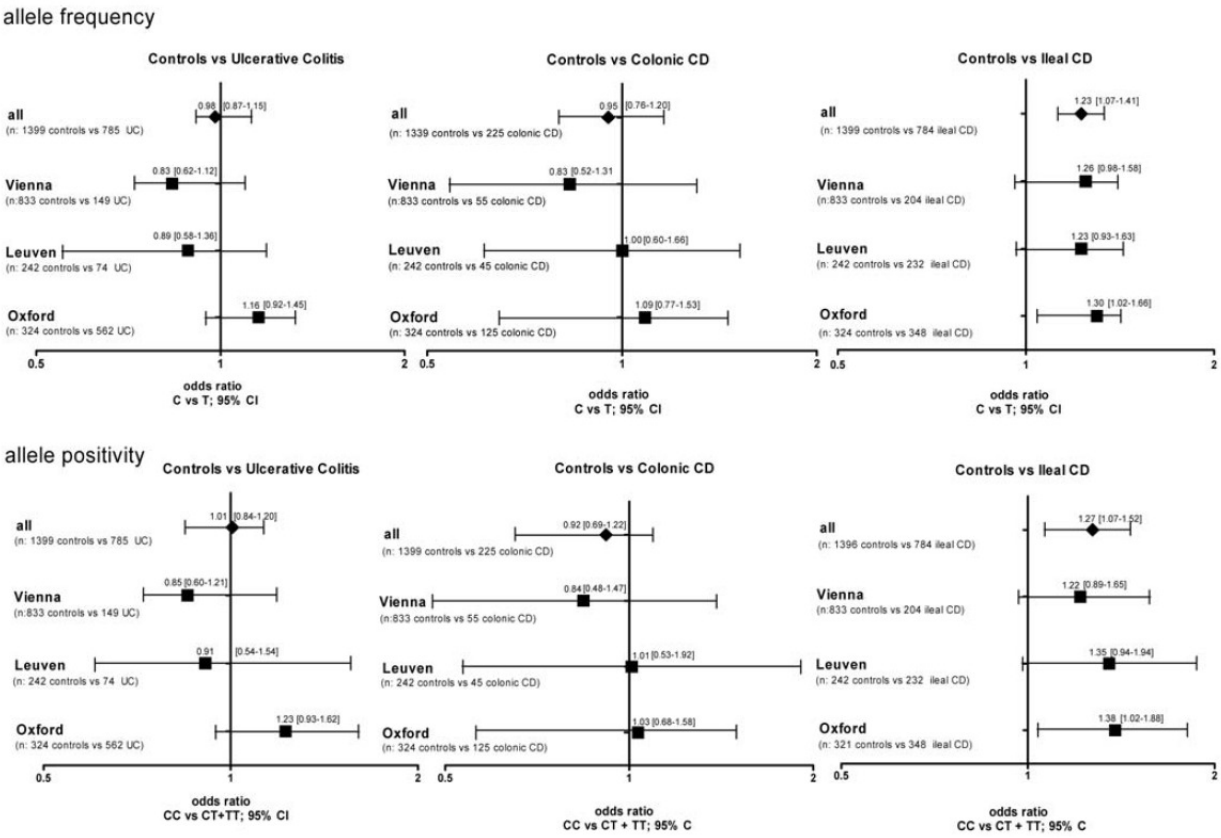

Figure 3. Differences in frequency distribution of rs3814570 in the different disease subgroups compared to healthy controls. Odds ratios and confidence intervals for the different comparisons are shown. The frequency distribution of rs3814570 was analyzed in different cohorts and combined samples: odds ratios and 95% confidence interval for allele frequency (upper panel) and allele positivity (amount of all T- allele carriers, lower panel) are shown for patients with ulcerative colitis (UC) (left panel), Crohn's disease (CD) patients with solely colonic involvement (L2, middle panel) and finally patients classified as either L1 (solely ileal) or L3 (ileal and colonic involvement) (right panel) compared to healthy control individuals.

doi:10.1371/journal.pone.0004496.g003

95% CI 0.91 to 1.57, p = 0.19618) and Vienna controls (allele frequency: OR 1.17, 95% CI 0.94 to 1.44, p = 0.15453), the differences did not achieve statistical significance. The elevated SNP frequency in ileal CD patients was seen in three independent European cohorts, and a distinct significant association of the minor variant for rs3814570 with ileal CD could be observed in the combined analysis of all samples (Table 2).

The association of rs3814570 with ileal CD is independent of gender but slightly more pronounced in patients >40 years

To make sure there is no disarrangement of age as well as gender we subgrouped all controls as well as the CD patient groups according to these criteria (Table 3). There were no consistent differences in allele frequency between men and women in either controls or patients; therefore we exclude a gender specific effect of the variant. Interestingly we found an increased association of the variant comparing patients with ileal, but not solely colonic CD of the age group A3 (>40 years) with controls of the same age group in the overall analysis, as well as in two separate cohorts (Leuven and Oxford). In the overall analysis a statistical significance for homozygous carriers was present (homozygous carriers: OR 2.02, 95% CI 1.01 to 4.05, p = 0.04347)

rs3814570 shows the highest frequency in patients with stricturing ileal Cohn's disease

We grouped the patients according to their behaviour into B1 (inflammatory), B2 (stricturing) and B3 (penetrating) (Table 4). We found the highest frequency in the overall analysis within the ileal CD subgroup with stricturing behaviour (T allele frequency: 31.25%). This was also obvious in 2 separate cohorts (T allele frequency in Oxford: 29.81%, Leuven: 35.83%) but not seen in L2 CD patients. The association of the SNP with stricturing ileal CD compared to healthy controls exhibited a high significance in the overall analysis (allele frequency: OR 1.32, 95% CI 1.08 to1.62, p = 0.00686) and an additionally increased amount of homozygous carriers was observed (homozygous carriers: OR 1.71, 95% 1.11 to 2.63, p = 0.01460). To identify a possible association with aggressiveness of disease we also grouped the patients in such that have had at least one surgery for CD and those who did not (Table 4). No consistent result was observed; even though in two cohorts a trend towards a higher frequency in the ileal CD group with surgery (T allele frequency in Oxford: 28.93% and Leuven:

Table 4. Patients sub grouped according to disease behaviour, L4 phenotype and surgery.

Disease behaviour and severity									
overall		**L1**	**L2**	**L3**	**ileal CD**	**CD**		**controls <> L1+L3**	
disease behaviour	**B1** (inflammatory)	17,12%	67,71%	22,20%	20,73%	31,31%			
	MAF	26,32%	24,17%	28,10%	27,67%	25,97%			
	B2 (stricturing)	50,90%	7,17%	27,71%	34,42%	28,28%	statistics for stricturing behaviour	**C<>T**	**CC<>CT+TT**
	MAF	30,97%	21,88%	31,46%	**31,25%**	30,71%		1.32; p = 0.00686	1.34; p = 0.02745
	B3 (penetrating)	31,98%	25,11%	50,09%	44,85%	40,40%		**Armitage's trend**	**CC<>TT**
	MAF	31,69%	26,79%	28,02%	28,78%	28,50%		1.30; p = 0.00963	1.71; p = 0.01460
upper GI involvement	**L4**	11,84%	8,00%	16,03%	14,80%	13,27%	statistics for L4	**C<>T**	**CC<>CT+TT**
	MAF	35,19%	22,22%	31,25%	**32,17%**	30,83%		1.38; p = 0.02882	1.50; p = 0.03749
	no L4	88,16%	92,00%	83,97%	85,20%	86,73%		**Armitage's trend**	**CC<>TT**
	MAF	29,60%	24,88%	29,18%	29,31%	28,25%		1.33; p = 0.03561	1.68; p = 0.10543
surgery	**at least one**	77,97%	33,33%	64,68%	68,54%	60,68%			
	MAF	30,79%	24,00%	29,11%	29,66%	28,97%			
	no	22,03%	66,67%	35,32%	31,46%	39,32%			
	MAF	29,00%	25,00%	29,59%	29,47%	27,78%			

MAF = minor allele frequency.
Shown are percentages of individuals per group as well as the *TCF-4* (*TCF7L2*) rs3814570 T- allele frequency (minor allele frequency MAF). Differences in genotype distribution compared to controls (all controls from Table 2) in general as well as the amount of all carriers (allele positivity) and the amount of homozygous carriers were subject to t- tests in patients with ileal CD. Finally, results of the Armitage's trend tests for verification of significant associations of the rare T- variant are shown.
doi:10.1371/journal.pone.0004496.t004

31.58%) and a significant stronger association with ileal CD in the surgery group compared to controls in one cohort (Oxford allele frequency: OR 1.34, 95% CI 1.03 to 1.74, p = 0.02885) was present.

rs3814570 confers to the risk of an additional L4 phenotype in patients with ileal CD

To specifically address the question of upper GIT involvement (L4) we separated the patient groups in further subgroups according to this specific additional phenotype. In general the amount of patients with upper GIT involvement was quite low: Leuven patients with additional L4 phenotype: 12 patients L3; 4 patients L2; 6 patients L1; Oxford patients with additional L4 phenotype: 36 patients L3; 4 patients L2; 10 patients L1; Vienna patients with additional L4 phenotype: 40 patients L3; 10 patients L2; 11 patients L1. Comparing the allele frequencies with controls, we found a slight increase in patients with ileal CD and additional L4 phenotype (T allele frequency: 32.17%). This did not account for L2 patients with upper GIT involvement. The stronger association of the rare variant was also statistically significant in the overall analysis (allele frequency: OR 1.38, 95% CI 1 1.03 to1.84, p = 0.02882).

rs3814570 is independent of *NOD2*

Given that the 3020insC frameshift mutation (SNP13) in *NOD2* is a known susceptibility factor for CD of the ileum and is associated with reduced HD-5 and-6 levels, we investigated if the observed association of rs3814570 with ileal CD is independent of *NOD2* in the Vienna and Leuven cohorts. We previously reported that the effects of reduced TCF-4 on Paneth cell α-defensins in ileal CD patients were independent of the effects of the SNP13 *NOD2* variant, since patients with this *NOD2* mutation showed a much more marked decrease of HD-5 and -6 expression [9]. The independence of the factors suggests that excluding patients harbouring *NOD2* SNP13 should yield similar allele frequencies of rs3814570 in the remaining ileal CD patients. Indeed, comparing all Leuven ileal CD patients (n = 232) to a subgroup excluding patients harbouring SNP13 (n = 191), there were no differences in allele frequency (OR 0.99) or allele positivity (OR 0.98). The same was true for the Vienna ileal CD patients (n = 204): following SNP13 exclusion (n = 154) the allele frequency gave an OR 1.06 and an allele positivity of OR 1.04. Thus, exclusion of patients with *NOD2* frameshift mutation SNP13 does not alter the observed allele frequencies of rs3814570 in patients with ileal CD, supporting independent effects of this *TCF-4* SNP and *NOD2* SNP13 in ileal CD.

Discussion

In a hypothesis driven candidate gene approach, we investigated the association of sequence polymorphisms in the *TCF-4* (*TCF7L2*) promoter with ileal Crohn's disease. The reported findings represent the third identified genetic association with a link to Paneth cells in ileal CD. Recently Cadwell et al. published that Crohn's disease patients homozygous for the disease risk allele of *ATG16L1* display Paneth cell abnormalities which were also present in ATG16L1^{HM} mice [23]. Earlier we and others have shown that the 3020insC (SNP13) mutation in the intracellular, in Paneth cell present muramyl dipeptide receptor *NOD2*, is associated with especially reduced levels of HD5 and -6 [7,8,12]. Such a distinct deficiency in the innate defence also characterizes *NOD2* knock out mice [24]. The characteristic decrease of HD-5 and -6 in ileal CD results in an impaired innate immunity at the small intestinal barrier which is distinguished by reduced antibacterial activity in the epithelium, and proposed to disrupt the host - microbe balance at

the mucosa [7,8,12]. It is also apparent in patients with wild type *NOD2* or either missense mutations (SNP8 and SNP12) but was, however, more pronounced in patients with the frameshift (SNP13) mutation. Different studies point to a delicate balance between commensal microbes and the intestinal mucosa (for review [25,26]). We propose that a perturbation in this dynamic interplay has an important role in IBD pathogenesis [27]. In summary, *NOD2* SNP13 can in part explain a loss in HD-5 and -6 level but is found in only a minority of patients with ileal CD, but diminished defensin levels are present in the majority [8] and have an immediate effect on antimicrobial activity against and composition of the intestinal microflora [8]. A different functional link in ileal CD leading to diminished Paneth cell α-defensins HD-5 and -6, is a reduced mRNA expression of *TCF-4*, which has been previously reported by our group [9]. The identification of *TCF-4* as a new factor in the pathogenesis of ileal CD provides a more general mechanism for the deficit in HD-5 and -6. Since TCF-4 binds to and directly regulates the promoter regions of *HD-5* and *HD-6*, diminished *TCF-4* expression, maybe consequent on a genetic mutation, could account for a decrease in both of these defensins. The current data give support to the hypothesis of a genetic association between a rare SNP variant of *TCF-4* and ileal involvement in CD in a subset of patients. This variant, the rs3814570 T allele in the *TCF-4* promoter region, was most prevalent in CD localized to the ileum and no association with either colonic CD or UC was found. The strongest association with the variant was present in ileal CD patients with stricturing disease behaviour as well as those with an additional involvement of the upper GIT. The fact that genetic variants in TCF-4, a factor indispensable for Paneth cell function, are specifically associated with ileal CD provides further evidence that a decrease in HD-5 and -6 is predisposing and can be seen as a primary defect in the disease. The reported association between the *TCF-4* SNP and small intestinal involvement was found in all cohorts from Vienna, Oxford and Leuven. Variability of allele frequencies in controls between the Oxford cohort and the two cohorts from the European mainland might be explained by population differences as a consequence of a heterogenic ethnical history. For *NOD2* an even greater heterogeneity among Europeans has been reported [28]. In a letter regarding the frequency variability of DLG5 polymorphisms, another gene reported to exhibit an association with IBD, Tenesa et al. caution against pooling data from different populations, because true but in the cohorts different effects might be concealed [29]. The overall analysis of controls versus ileal CD showed a marginally lower difference when compared with the individual results of the Vienna and Oxford cohorts (Figure 3), so this might play a role in our analysis. However, differences in overall allele frequencies in the control cohorts were not statistically different.

Associations of genetic variations of the *TCF-4* gene with other diseases exist, but data are limited. An association of two non-coding SNPs in the *TCF-4* gene has been observed with diabetes mellitus [30] and, in another study, an association with deletions and insertions of adenines in the coding region was reported in patients with colorectal cancer [22]. We did not find any genetic association of these polymorphisms in UC nor CD (or in any of the clinical subgroups) in the samples from Vienna (Figure 2a and data not shown for repetitive A polymorphic region).

Given that Wnt/TCF-4 plays a major role in Paneth cell maturation, aside from its direct function in the expression of Paneth cell α-defensins [31,32], the observed link between ileal CD and *TCF-4* suggests that impaired cell differentiation might be involved in the disorder. This would differ from many other views on IBD pathogenesis which emphasize the role of dysregulated immune function in otherwise normally functioning cells. If indeed a hypothesis on aberrant cell maturation proves significant, effective new therapeutic strategies might alternatively target steps in differentiation in addition to regulate or influence downstream impaired effector molecules like HD-5 and -6.

Supporting Information

Figure S1 Sequencing of *TCF-4* (*TCF7L2*) exon regions and intron boundaries. Sequencing of exon regions was performed in a representative and limited number of healthy controls as well as Crohn's disease patients with known clinical phenotype (small intestinal CD). The relative location of identified variants is marked via grey dashes (upper part) and their allele frequency is demonstrated via bars for controls as well as patients (lower part). P<0,05 is considered statistical significant.
Found at: doi:10.1371/journal.pone.0004496.s001 (10.07 MB DOC)

Table S1 *TCF-4* (*TCF7L2*) rs3814570 frequency distribution and statistical analysis of Oxford cohort samples. The different distribution of genotypes is demonstrated for each group and subgroup: controls, inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), CD with solely colonic involvement (L2) and CD with solely ileal (L1), and ileo-colonic CD (L3). Differences in genotype distribution compared to controls as well as the number of carriers (allele positivity) were subject to t-tests. Results of the Armitage's trend tests for verification of significant associations with the minor T- variant are shown.
Found at: doi:10.1371/journal.pone.0004496.s002 (0.05 MB DOC)

Table S2 *TCF-4* (*TCF7L2*) rs3814570 frequency distribution and statistical analysis of Vienna cohort samples. The different distribution of genotypes is shown for each group and subgroup: controls, inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), CD with solely colonic involvement (L2), CD with solely ileal (L1) and ileo-colonic CD (L3). Differences in genotype distribution compared to controls as well as the number of carriers (allele positivity) were subject to t- tests as well as Armitage's trend test.
Found at: doi:10.1371/journal.pone.0004496.s003 (0.05 MB DOC)

Table S3 *TCF-4* (*TCF7L2*) rs3814570 frequency distribution and statistical analysis of Leuven cohort samples. The different distribution of genotypes is demonstrated for each group and subgroup: controls, inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC), CD with solely colonic involvement (L2), CD with solely ileal (L1) and ileo-colonic CD (L3). Differences in genotype distribution compared to controls as well as the number of carriers (allele positivity) were subject to t-tests as well as Armitage's trend test.
Found at: doi:10.1371/journal.pone.0004496.s004 (0.05 MB DOC)

Acknowledgments

We thank Drs. C. Schaefer, K. Rotfuß, H.P. Kreichgauer and others from the Robert Bosch Hospital for help with sample procurement. We thank K. Siegel, J. Bader and A. Zwicker for excellent technical assistance. We also thank M. Eichelbaum for discussion and support.

Author Contributions

Conceived and designed the experiments: MJK IK MC ES WR GW PR SV RC KF DJ CB MS EFS JW. Performed the experiments: MJK IK GW. Analyzed the data: MJK IK MC ES WR GW JB AT LPB SW DJ MS EFS JW. Contributed reagents/materials/analysis tools: ES WR AT KRH PR SV RC KF DJ MS JW. Wrote the paper: MJK JB CB JW.

References

1. Podolsky DK (2002) Inflammatory bowel disease. N Engl J Med 347: 417–429.
2. Strober W (2006) Immunology. Unraveling gut inflammation. Science 313: 1052–1054.
3. Wehkamp J, Schmid M, Fellermann K, Stange EF (2005) Defensin deficiency, intestinal microbes, and the clinical phenotypes of Crohn's disease. J Leukoc Biol 77: 460–465.
4. Louis E, Collard A, Oger AF, Degroote E, Aboul Nasr El Yafi FA, et al. (2001) Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. Gut 49: 777–782.
5. Gasche C, Grundtner P (2005) Genotypes and phenotypes in Crohn's disease: do they help in clinical management? Gut 54: 162–167.
6. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, et al. (2005) Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 19 Suppl A: 5–36.: 5–36.
7. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schaeffeler E, et al. (2004) NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal a-defensin expression. Gut 53: 1658–1664.
8. Wehkamp J, Salzman NH, Porter E, Nuding S, Weichenthal M, et al. (2005) Reduced Paneth cell {alpha}-defensins in ileal Crohn's disease. PNAS 102: 18129–18134.
9. Wehkamp J, Wang G, Kübler I, Nuding S, Gregorieff A, et al. (2007) The Paneth Cell {alpha}-Defensin Deficiency of Ileal Crohn's Disease Is Linked to Wnt/Tcf-4. J Immunol 179: 3109–3118.
10. Kiehne K, Brunke G, Wegner F, Banasiewicz T, Folsch UR, et al. (2006) Defensin expression in chronic pouchitis in patients with ulcerative colitis or familial adenomatous polyposis coli. World J Gastroenterol 12: 1056–1062.
11. Fishbein T, Novitskiy G, Mishra L, Matsumoto C, Kaufman S, et al. (2008) NOD2-expressing bone marrow-derived cells appear to regulate epithelial innate immunity of the transplanted human small intestine. Gut 57: 323–330.
12. Elphick D, Liddell S, Mahida YR (2008) Impaired Luminal Processing of Human Defensin-5 in Crohn's Disease: Persistence in a Complex with Chymotrypsinogen and Trypsin. Am J Pathol 172: 702–713.
13. van Es JH, Jay P, Gregorieff A, van Gijn ME, Jonkheer S, et al. (2005) Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat Cell Biol 7: 381–386.
14. Hayward P, Kalmar T, Arias AM (2008) Wnt/Notch signalling and information processing during development. Development 135: 411–424.
15. Gregorieff A, Clevers H (2005) Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev 19: 877–890.
16. Gregorieff A, Pinto D, Begthel H, Destree O, Kielman M, et al. (2005) Expression Pattern of Wnt Signaling Components in the Adult Intestine. Gastroenterology 129: 626–638.
17. Korinek V, Barker N, Moerer P, van DE, Huls G, et al. (1998) Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 19: 379–383.
18. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, et al. (2002) Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 111: 251–263.
19. Welcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661–678.
20. Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, et al. (2004) Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut 53: 987–992.
21. Fellermann K, Stange DE, Schaeffeler E, Schmalzl H, Wehkamp J, et al. (2006) A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. Am J Hum Genet 79: 439–448.
22. Duval A, Gayet J, Zhou XP, Iacopetta B, Thomas G, et al. (1999) Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. Cancer Res 59: 4213–4215.
23. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, et al. (2008) A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 456: 259–263.
24. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, et al. (2005) Nod2-Dependent Regulation of Innate and Adaptive Immunity in the Intestinal Tract. Science 307: 731–734.
25. Hooper LV, Gordon JI (2001) Commensal host-bacterial relationships in the gut. Science 292: 1115–1118.
26. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. Science 307: 1915–1920.
27. Schreiber S, Rosenstiel P, Albrecht M, Hampe J, Krawczak M (2005) Genetics of Crohn disease, an archetypal inflammatory barrier disease. Nat Rev Genet 6: 376–388.
28. Medici V, Mascheretti S, Croucher PJ, Stoll M, Hampe J, et al. (2006) Extreme heterogeneity in CARD15 and DLG5 Crohn disease-associated polymorphisms between German and Norwegian populations. Eur J Hum Genet 14: 459–468.
29. Tenesa A, Noble C, Satsangi J, Dunlop M (2006) Association of DLG5 and inflammatory bowel disease across populations. Eur J Hum Genet 14: 259–260.
30. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, et al. (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet 38: 320–323.
31. Wehkamp J, Chu H, Shen B, Feathers RW, Kays RJ, et al. (2006) Paneth cell antimicrobial peptides: Topographical distribution and quantification in human gastrointestinal tissues. FEBS Lett 580: 5344–5350.
32. George MD, Wehkamp J, Kays RJ, Leutenegger CM, Sabir S, et al. (2008) In vivo gene expression profiling of human intestinal epithelial cells: analysis by laser microdissection of formalin fixed tissues. BMC Genomics 9: 209.

3.2. Association of a functional variant in the Wnt co- receptor LRP6 with early onset ileal Crohn's disease

During the review of this thesis the following data were under revision for publication at PLoS Genetics. They are now available online within "Koslowski MJ et al. (2012) Association of a Functional Variant in the Wnt Co-Receptor LRP6 with Early Onset Ileal Crohn's Disease. PLoS Genet 8(2): e1002523"

Background

Focusing primarily on and selecting LRP6 as an ileal CD candidate gene was hypothesis driven and based on literature search. As explained in the introduction, accumulation of β-catenin is the essential step in canonical Wnt signaling. It greatly depends on the unrestricted function of LRP6, a key factor in the transduction of the canonical Wnt signal in the intracellular cascade via interaction with various pathway factors in multiple ways (Holmen et al., 2002; Tamai et al., 2000; Bafico et al., 2001). The characterization of LRP6 as a canonical Wnt signaling component is a quite young discovery but ever since, more than 200 publications highlight either its crucial function in the pathway, the regulation of its activity, interaction with other factors or its involvement in diseases. Different model systems could demonstrate a decisive position of the co-receptor in Wnt signaling and support a broad role in the development and homeostasis of different tissues. Mice homozygous for a LRP6 insertion mutation exhibit developmental defects strikingly similar to those caused by mutations in individual Wnt genes (Pinson et al., 2000). Other LRP6 mutations are followed by dorsal thalamic developmental defects (Zhou et al., 2004), reduced terminal end buds and branches during postnatal development (Lindvall et al., 2009), as is the full knockout by a delay in dopaminergic neuron differentiation (Castelo-Branco et al., 2009). Its inactivation results in a block in the orofacial primordial, which might be an underlying mechanism for cleft lip formation (Song et al., 2009). Mice with a spontaneous hypomorphic LRP6 point mutation (ringelschwanz mutation) furthermore exhibit different skeletal and neural tube abnormalities (Kokubu et al., 2004). As already mentioned in the introduction, a tissue specific *in vivo* inactivation of the receptor causes heavy changes in the intestinal mucosal architecture with serious

and harmful consequences in mice. Besides being studied in different models, LRP6 has also been investigated in different disease settings.

Overview on disease associations of LRP6

Disease	Defect/Event	Comprehensive disorder	Reference
Late onset Alzheimer's	Association of common LRP6 SNPs	Degenerative disorder	(De Ferrari et al., 2007)
Age- related macular degeneration	Association of LRP6 genetic variants	Degenerative disorder	(Haines et al., 2006)
Idiopathic pulmonary fibrosis	Increase in activated (Phospho-) LRP6	Chronic progressive disease/ Inflammatory disorder	(Konigshoff et al., 2008)
hypercholesterolemia and early-onset atherosclerosis	Association of LRP6 mutation (R611C)	Cardiovascular disease/ Impaired cellular LDL clearance	(Liu et al., 2008)
Autosomal dominant early coronary disease	Mutations in LRP6	Cardiovascular disease	(Mani et al., 2007)
Carotid artery atherosclerosis	Association of coding LRP6 variant (1062V)	Cardiovascular disease	(Sarzani et al., 2009)
Osteoporosis, osteoarthritis, low or high bone mass density	Loss- or gain-of-function mutations in LRP6	Bone disease	(Williams and Insogna, 2009)

Table.3.1: Publications dealing with an involvement of LRP6 in different diseases ranging from degenerative disorders to cardiovascular, fibrosing and bone diseases. LRP6 defects include either functional mutations in the receptor or association of non coding genetic variants as well as an increased activation in Idiopathic pulmonary fibrosis.

In the herein presented study, the distribution of coding LRP6 SNPs was now analyzed in an IBD cohort from Oxford. Prospective testing was performed in 2 additional European sample sets. The main scientific aim was to identify functional variants with a potential influence on Wnt pathway activity and as a consequence Paneth cell α-defensin expression. The assessment of ileal LRP6 expression level in healthy controls as well as patients was also planned and biopsy mRNA isolates from genotyped healthy controls (n=22) as well as CD patients with ileal (n=35) or isolated colonic involvement (n=12) were selected. It was also planned to analyze the functional relevance of hypothetically associated coding SNPs in the same sample set from Stuttgart.

Results

The Wnt co- receptor LRP6 could be identified as a novel and relevant factor in CD with small intestinal involvement. The co- receptor shows impairments on multiple levels, allowing a division of the results of the study in three main parts

1. A coding LRP6 SNP is associated with early onset ileal CD

Distribution of exonic LRP6 SNPs in a large Cohort from Oxford

Frequency distributions and linkage disequilibria of prioritized exonic LRP6 SNPs (see also chapter 2.8.2.3, Fig. 2.1) were analyzed in a well defined cohort from Oxford which included almost 2000 DNA samples grouped in healthy controls and IBD patients. Frequencies could be determined for 5 of the 12 exonic SNPs described in the NCBI SNPdb (Figure 3.1 lower panel). SNPs with a minor allele frequency (MAF) of 0 in the Oxford samples were also either not previously validated according to information in the SNPdb or they were not found in individuals with western European descent. None of the SNPs tested were associated with CD or UC overall. However, the coding minor allele of rs2302685 (GTT <> ATT / Ile1062Val) exhibited a specific association with the early disease onset subgroup in ileal CD (OR: 1.524, 95%, CI 0.988 to 2.345, p=0.05511; for homozygous carriers OR 3.152, 95% CI 1.128 to 8.845, p=0.02144) in this first analysis. Since none of the other analyzed SNPs showed frequency differences between controls and the different disease groups we focused only on rs2302685 for additional tests. We also did not find a significant degree of linkage between this variant and any of the other tested polymorphic SNPs (Figure 3.1 lower panel) and therefore didn't include them in subsequent testing.

		Overview groups				*Overview ileal CD*						
Oxford			**IBD**			**Gender**		**Disease behavior**			**Age at diagnose**	
		controls	*UC*	*colonic CD*	*ileal CD*	*male*	*female*	*B1*	*B2*	*B3*	*>17*	*</= 17*
	TT	470	416	89	238	90	134	33	109	**82**	207	**31**
Genotypes	CT	242	185	53	123	50	71	18	54	**49**	102	**21**
(n)	CC	24	33	4	16	8	8	2	4	**10**	11	**5**
samples (n)	all	736	634	146	377	148	213	53	167	**141**	320	**57**
Genotypes	TT	63.86	65.62	60.96	63.13	60.81	62.91	62.26	65.27	**58.16**	64.69	**54.39**
(%)	CT	32.88	29.18	36.3	32.63	33.78	33.33	33.96	32.34	**34.75**	31.88	**36.8**
	CC	3.26	5.21	2.74	4.24	5.41	3.76	3.77	2.4	**7.09**	3.44	**8.77**
Allele	T	80.3	80.21	79.11%	79.44	77.7	79.58	79.25	81.44	**75.53**	80.63	**72.81**
Frequency (%)	C	19.7	19.79	20.89	20.56	22.3	20.42	20.75	18.56	**24.47**	19.38	**27.19**

Table 3.2: **LRP6 rs2302685 frequency distribution in the oxford cohort samples:** the different distribution of genotypes is demonstrated for each group and subgroup: controls, ulcerative colitis (UC), Crohn's Disease (CD) with solely colonic involvement (colonic CD; L2) and CD with solely ileal as well as ileal and colonic involvement (ileal CD; L1+L3). The ileal CD group was further sub grouped according to gender, disease behavior (inflammatory B1; stricturing B2; penetrating B3) and age at diagnosis (older than 17: >17; 17 and younger: </=17)

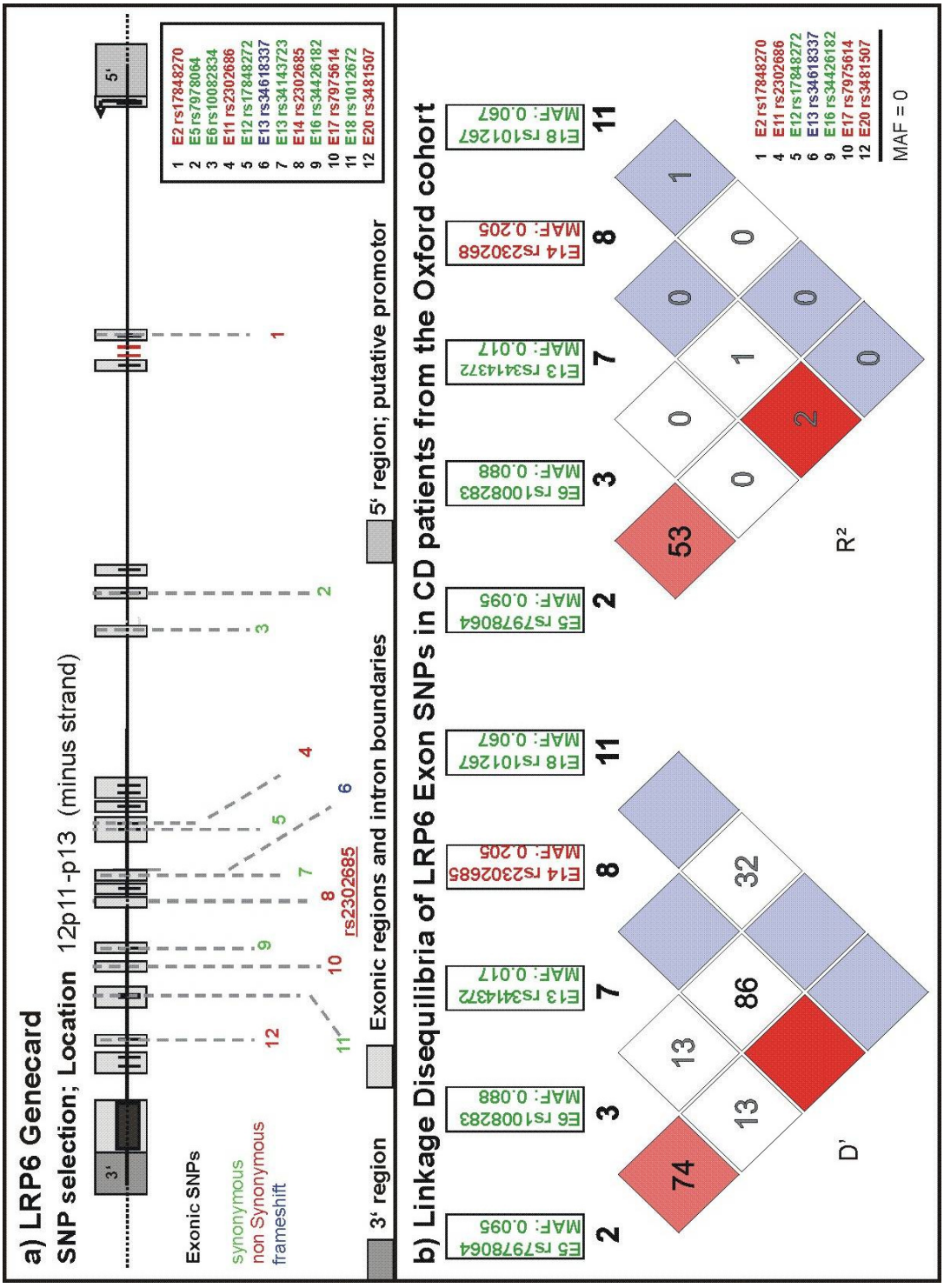

Figure 3.1: **Known exonic SNPs in LRP6**: Upper panel (a): LRP6 gene card with locations of exonic SNPs published in the NCBI SNPdb. Variants which lead to an amino acid exchange are marked in red. A deletion mutation causing a different protein chain is marked in blue, silent variants are written in green. Lower pane (b)l: Linkage disequilibria (LD) of the exonic SNPs in CD patients of the Oxford cohort. 7 of the genotyped SNPs were not present. We conclude that there is no significant LD between the sole coding variant (rs2302685) and any of the synonymous SNPs.

Consistent with the initial finding, the subsequent analysis of two additional cohorts from Leuven and Vienna (see appendix for numbers and genotyping frequencies) reproduced the specific association, whereas frequency distributions among the control groups as well as the ungrouped IBD patients were strikingly similar. The associated variant is known to reduce canonical Wnt activity as it is buried in and functionally interferes with the hydrophobic core of the receptors b-propeller domain (De Ferrari et al., 2007).

		Overview groups				*Overview ileal CD*						
all samples			**IBD**			**Gender**		**Disease behavior**			**Age at diagnose**	
		controls	*UC*	*colonic CD*	*ileal CD*	*male*	*female*	*B1*	*B2*	*B3*	*>17*	*≤ 17*
	TT	1277	758	147	500	219	267	98	183	**198**	452	**48**
Genotypes	CT	609	364	83	263	112	152	58	76	**126**	228	**35**
(n)	CC	65	47	7	31	15	16	5	9	**16**	21	**10**
samples (n)	all	1951	1169	237	794	346	435	161	268	**340**	701	**93**
	TT	65.45	64.84	62.03	62.97	63.29	61.38	60.87	68.28	**58.24**	64.48	**51.61**
Genotypes	CT	31.21	31.14	35.02	33.12	32.37	34.94	36.02	28.36	**37.06**	32.52	**37.63**
(%)	CC	3.33	4.02	2.95	3.9	4.34	3.68	3.11	3.36	**4.71**	3	**10.75**
Allele	T	81.06	80.41	79.54	79.53	79.48	78.85	78.88	82.46	**76.76**	80.74	**70.43**
Frequency	C	18.94	19.59	20.46	20.47	20.52	21.15	21.12	17.54	**23.24**	19.26	**29.57**

Table 3.3: **LRP6 rs2302685 frequency distribution in all samples:** the different distribution of genotypes is demonstrated for each group and subgroup: controls, ulcerative colitis (UC), Crohn's Disease (CD) with solely colonic involvement (colonic CD; L2) and CD with solely ileal as well as ileal and colonic involvement (ileal CD; L1+L3). The ileal CD group was further sub grouped according to gender, disease behavior (inflammatory B1; stricturing B2; penetrating B3) and age at diagnosis (older than 17: >17; 17 and younger: </=17)

The distribution of rs2302685 within all displayed groups among all cohorts as well as within the overall samples was tested for deviation from Hardy Weinberg (HW) equilibrium and for association via freely available software as stated in the methods part. We found no significant divergence from HW equilibrium as tested by Pearson's goodness-of-fit chi-square test. The calculated frequencies and test results for the comparison of all controls with early onset CD patients are in detail stated below.

All controls:

Genotype TT: n = 1277 (1281.98) [observed (expected)] chi^2= 0.019

Genotype CT: n = 609 (599.04) [observed (expected)] chi^2= 0.166

Genotype CC: n = 65 (69.98) [observed (expected)] chi^2= 0.354

Allele frequency estimates and standard deviation:

Allele T: 3163/3902 = 0.811 +/- 0.0062

Allele C: 739/3902 = 0.189 +/- 0.0062

Deviation from HW equilibrium:

Inbreeding coefficient (defined as the probability that the alleles at the particular locus from two individuals are identical by descent): F = -0.01663

Pearson's goodness-of-fit chi-square: chi^2= 0.539, p= 0.46274 (establishes whether or not the observed frequency distribution differs from a theoretical distribution)

All early onset ileal CD patients:

Genotype TT: n = 48 (46.13) [observed (expected)], chi^2= 0.076

Genotype CT: n = 35 (38.74) [observed (expected)], chi^2= 0.360

Genotype CC: n = 10 (8.13) [observed (expected)], chi^2= 0.429

Allele frequency estimates and standard deviation:

Allele T: 131/186 = 0.704 +/- 0.0350

Allele C: 55/186 = 0.296 +/- 0.0350

Deviation from HW equilibrium:

Inbreeding coefficient: F = 0.09646

Pearson's goodness-of-fit chi-square: chi2= 0.865, P= 0.35225

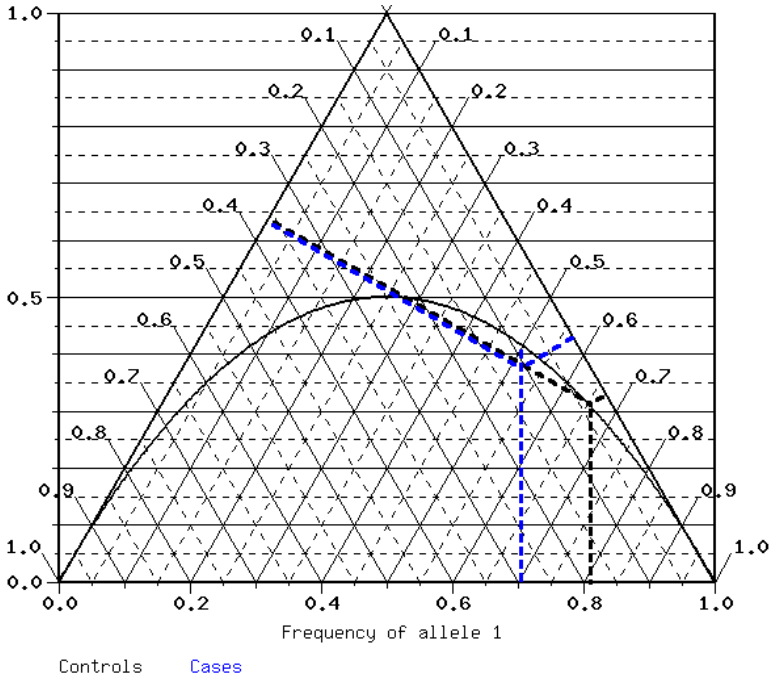

Figure 3.2: De Finetti diagram with Hardy Weinberg parabola. Early onset ileal Crohn's Disease patients are marked in blue; the controls are displayed in black. The X-axis represents the frequency of Allele T, the intersection of the Hardy Weinberg parabola and the vertical line corresponds to the frequency of the heterozygous genotype (CT) in case of a perfect Hardy-Weinberg equilibrium. The diagram length of the vertical line hitting the two perpendicular lines displays the actual frequency of the genotype CT. The length of left perpendicular line marks the frequency of homozygous wildtype individuals (TT). The length of the right perpendicular line stands for the frequency of the CC genotype.

In the combined samples, a quite strong association with early onset disease (OR 1.797, 95% CI 1.298 to 2.486, p=0.00034) and a weaker one with penetrating behavior (OR 1.296, 95% 1.066 to 1.575, p=0.00917) in the ileal CD subphenotype hints to an influence of the mutation in disease onset and severity, even though the latter association lost its significance after adjusting for multiple testing (Bonferroni adjustment for penetrating ileal CD behavior: p=0,10087). Gender on the other hand had no impact on the allele distribution (Table 3.3). The homozygous genotype of the minor allele displayed the highest risk for early onset ileal disease underlining a potential dose effect of the mutation (homozygous minor allele carriers: controls: 3,33%, early onset ileal CD: 10.75%; OR 4.093, 95% CI 1.981 to 8.455, p=0.00004). Amongst the 237 analyzed patients with exclusive colonic CD (L2) only 19 had a disease

onset prior to age 18. None of these were homozygous for the risk variant and with a MAF of 13,64%, the SNP distribution showed no significant difference to controls (OR 0.803, 95% CI 0.334 to 1.926, p=0.62172). We also compared early versus late onset in ileal CD patients and found a similar result as in the comparison with healthy controls (allele frequency: OR 1.760, 95% CI. 1.251 to 2.477, p=0.00106; homozygous carriers: OR 4.484, 95% CI 1.995-10.077, p=0.00009). The mean age of onset was similar between the CD patients in the different cohorts, as was the mean
age of controls at the time of blood sampling for later DNA extraction (Table 3.4).

	Mean age at sampling	*Mean age at onset*	
	Controls	**Ileal CD (L1+L3)**	**Colonic CD (L2)**
Oxford	42.69 (± 14.62)	27.46 (± 11.79)	34.03 (± 14.71)
Leuven	35.00 (± 17.30)	30.33 (± 11.16)	31.97 (± 11.18)
Vienna	36,73 (± 11.99)	27.89 (± 9.39)	28,07 (± 10,12)

Table 3: Age of included individuals: Provided are the mean age ± standard deviation.

In addition to testing for allele frequency differences and the increased risk of homozygous carriers, we also used additive, recessive and dominant models of inheritance to compare the genotype distribution between controls and early onset ileal CD as presented in Table3.4.

Controls vs early onset ileal CD			Allele positivity		Armitage's trend
	allele frequency	**homozygous risk**	**dominant model**	**recessive model**	**additive model**
	C vs **T**	CC vs **TT**	CC vs **CT+TT**	TT vs **CT+CC**	**T** common odds
Odds ratio	**1.797**	**4.093**	*1.776*	**0.286**	**1.877**
95% CI	1.298 to 2.487	1.981 to 8.455	1.170 to 2.696	0.142 to 0.577	
p-value	0.00034	0.00004	0.00632	0.0002	0.00033
Bonferroni adjusted p-value	**0.00374**	**0.00044**	*0.06952*	**0.0022**	**0.00363**

Table 4: Overall statistical analysis in controls versus early onset ileal CD. The genotypes for which the risk was calculated are marked in bold. The Bonferroni adjusted p-values were calculated with regards to all comparisons in the overall sample group (adjusted for 11 tests).

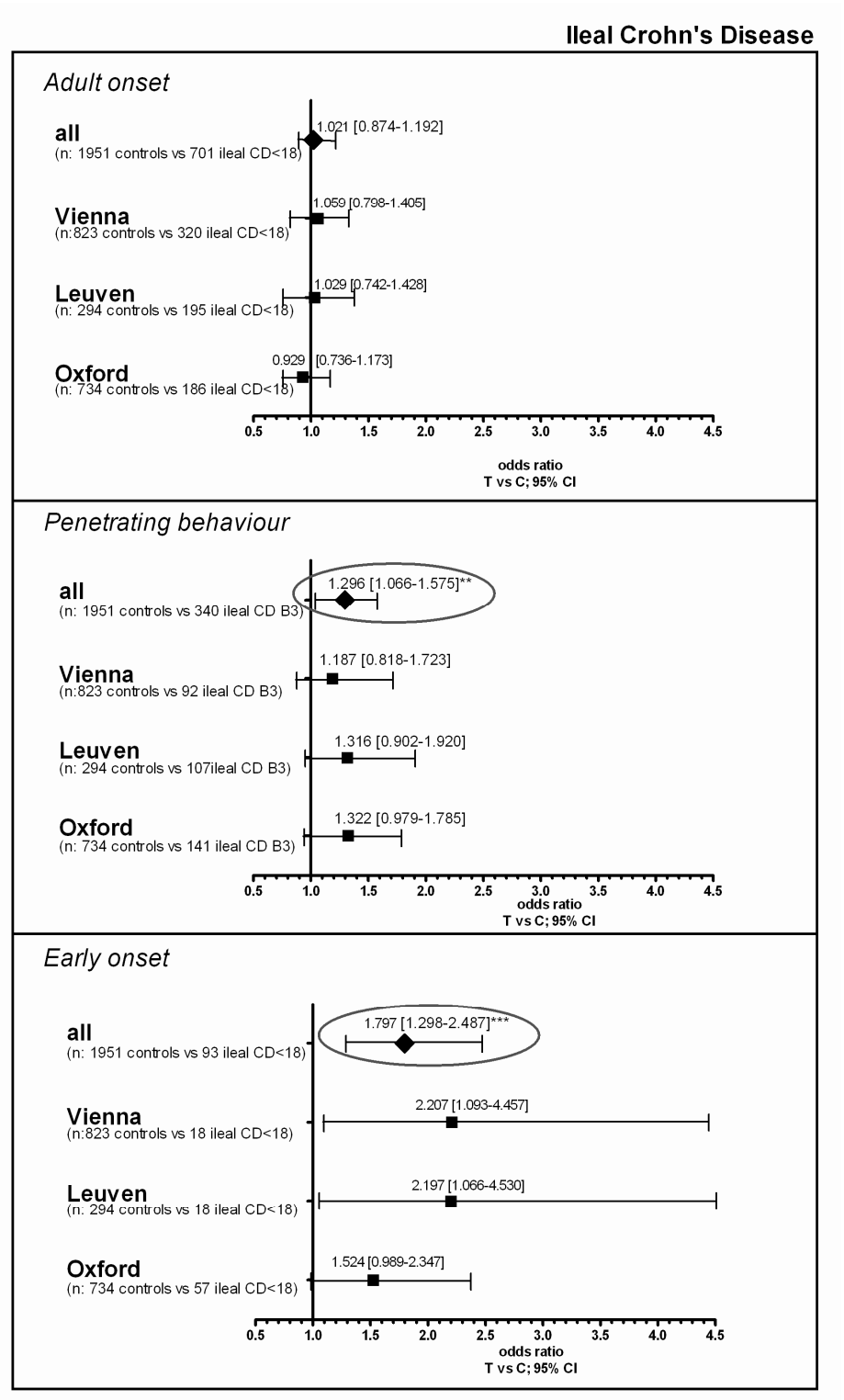

Figure 3.3: r**isk for rs2302685 C-allele carriers in the different ileal disease subgroups and the influence of detailed phenotyping.** Odds ratios and confidence intervals for the different comparisons are shown. The frequency distribution of the minor rs2302685 allele was analyzed in different cohorts and combined samples: ORs and 95% CI for the allele frequency are shown for patients with specific disease subtypes of ileal CD (classified as either L1 (solely ileal) or L3 (ileal and colonic involvement)) compared to healthy control individuals. The early onset as well as the penetrating behavior subgroups display a

significantly increased minor allele frequencies (MAF) whereas the variant does not increase the risk for adult ileal CD in general (OR = 1,01 in all samples).

Performing analysis for the different models of inheritance we found a dose dependency of the variant in early onset ileal CD, highlighting the high risk in homozygous carriers.

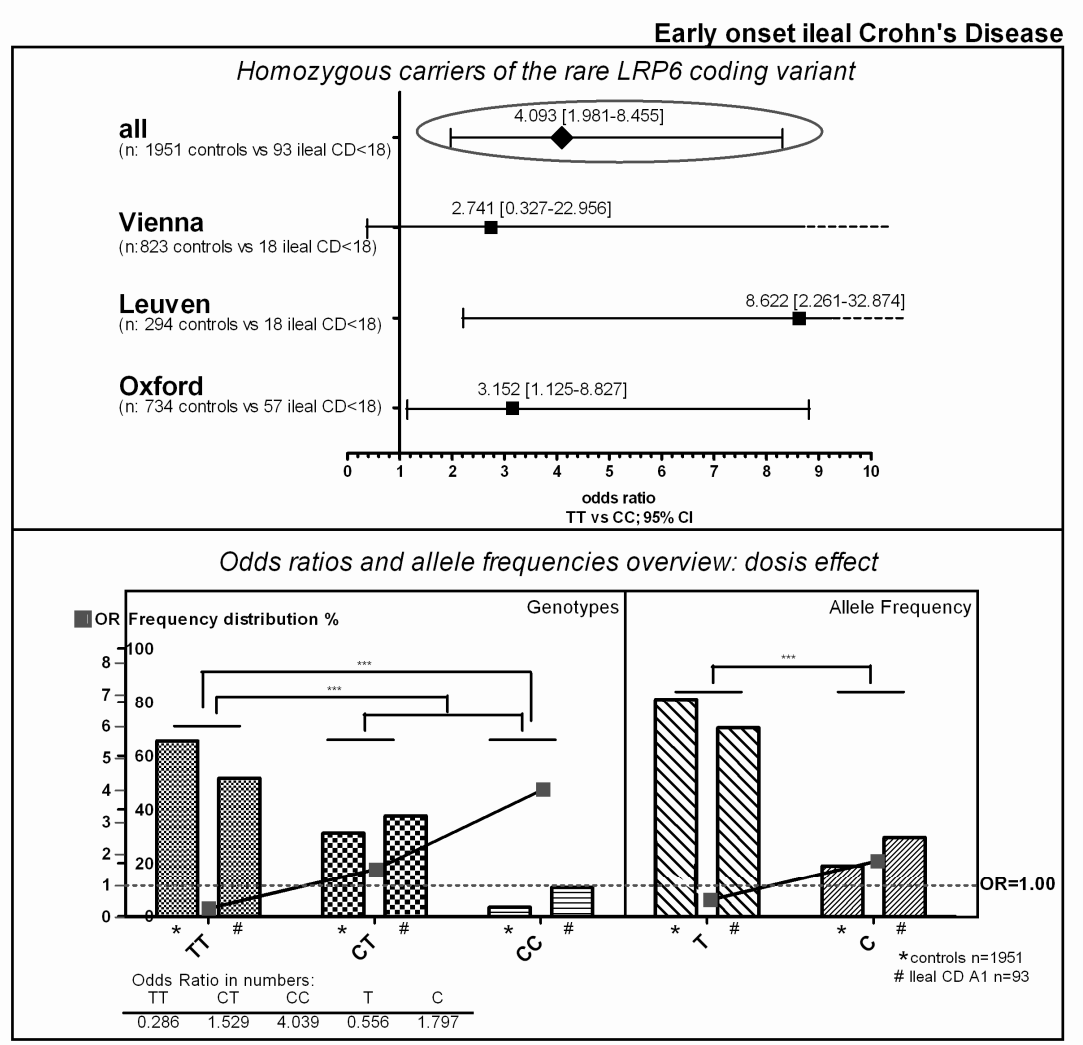

Figure 3.4**: An especially high risk for early onset ileal CD is found for homozygous rs2302685 C - allele carriers:** This becomes apparent in the high OR for such individuals in the different cohorts and combined samples (upper panel). The frequency difference between controls and early onset patients and the ORs for the different genotypes (TT, CT or CC) reveal a dose effect of the C-allele (lower panel).

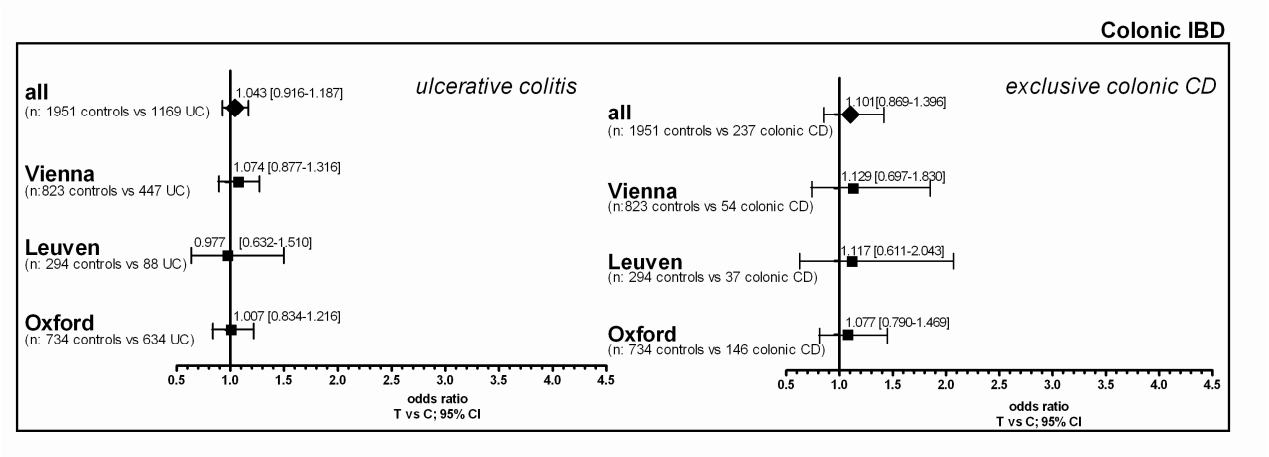

Figure 3.5: **The minor LRP6 variant is not associated with colonic forms of IBD**. Odds ratios and 95% confidence interval for the MAF are shown for patients with ulcerative colitis (UC) (left panel) and CD patients with solely colonic involvement (right panel) compared to healthy control individuals.

2. HD5 mRNA level seems further reduced in LRP6 mutated ileal CD patients

To study the functional consequences of the Ile1062Val mutation on Paneth cell antimicrobial efficacy, HD5 mRNA expression levels were analyzed in genotyped individuals. To exclude a bias due to influences of *NOD2* defects (Wehkamp et al., 2004b), individuals with mutations in the pattern recognition receptor were excluded from the analysis. Unfortunately in the available cohort we had no healthy controls or patients who carried the minor variant on both chromosomes but only homozygous wildtype and heterozygous individuals. The latter were defined as C-allele carriers and compared to those homozygous for the more common ancestral allele. Besides confirming the decrease of HD5 in ileal CD patients, a *LRP6* genotype dependent reduction of HD5 in the ileal CD patients who carried the rare SNP variant was found. Separation according to exclusive ileal (L1) and ileocolonic (L3) subphenotypes revealed no difference between the 2 groups in the affected C-allele carriers.

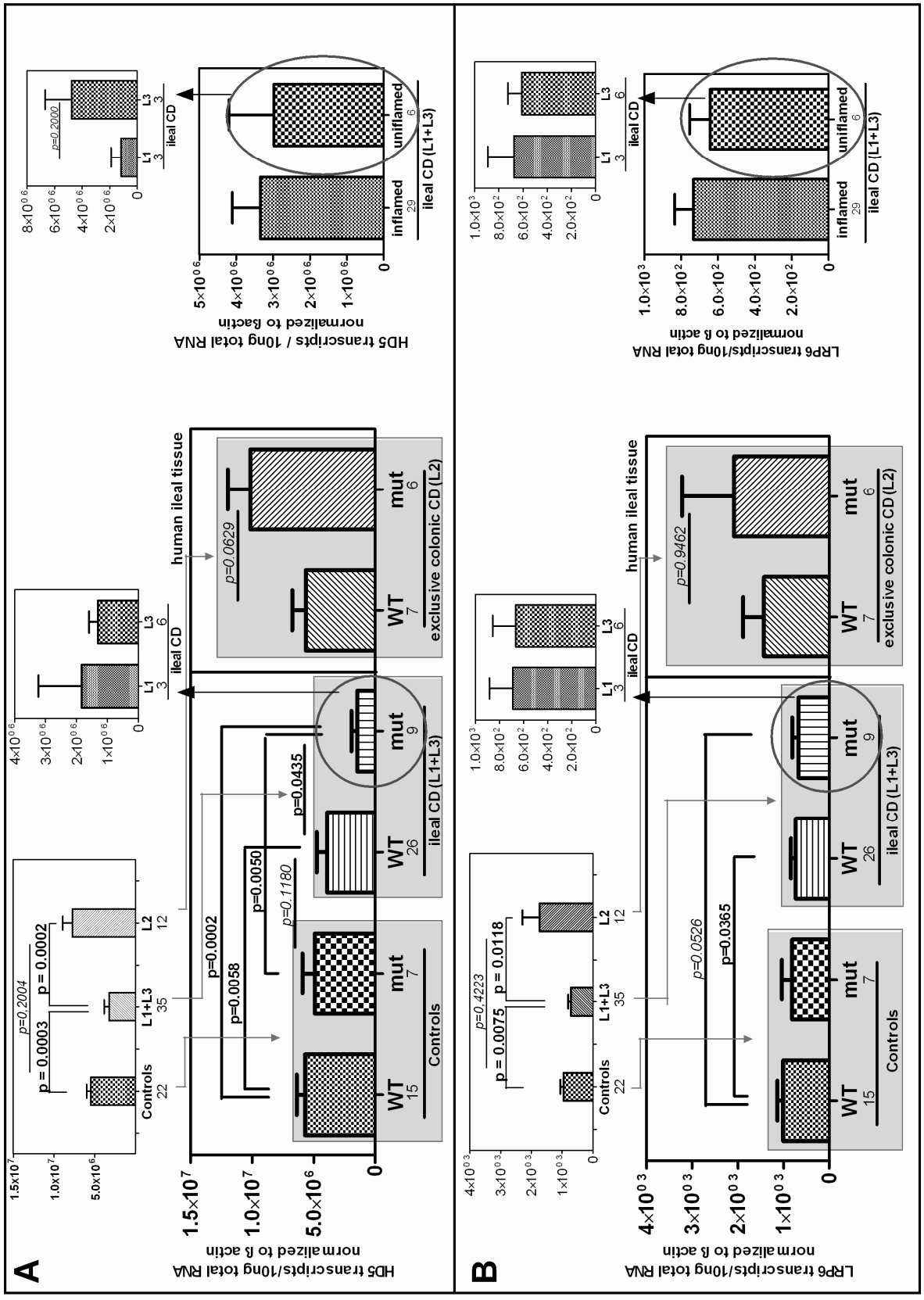
A
HD5 transcripts/10ng total RNA normalized to ß actin
human ileal tissue
Controls
ileal CD (L1+L3)
exclusive colonic CD (L2)
WT
mut
p=0.0002
p=0.0058
p=0.0050
p=0.1180
p=0.0435
p=0.0629
HD5 transcripts / 10ng total RNA normalized to ßactin
inflamed
uniflamed
ileal CD
B
LRP6 transcripts/10ng total RNA normalized to ß actin
p=0.4223
p = 0.0075
p = 0.0118
p=0.0526
p=0.0365
p=0.9462
LRP6 transcripts/10ng total RNA normalized to ß actin

Figure 3.6: **LRP6 and HD5 mRNA in ileal CD and the effect of the coding minor rs2302685 variant on their expression levels**. Taken all samples together, HD5 mRNA level are, as expected, diminished in ileal CD (A, upper figure). Analyzing the mRNA expression of LRP6 in the arranged cohort we also found a significant decrease of the factor in all patients with an ileal CD phenotyped compared to healthy individuals and colonic CD patients, mirroring the expression pattern of HD5 (B, upper figure). Arranging the ileal CD patients according to the inflammatory status, we found no difference in the 6 individuals who had no macroscopic inflammation at the time of biopsy sampling (A and B, right panels respectively). In contrast, the mRNA expression of HD5 seems to be influenced by the coding minor LRP6 rs2302685 allele (C- allele) in ileal CD patients (A). All cohort samples were sub grouped according to disease and analyzed according to the LRP6 genotype (WT for individuals homozygous for the common SNP variant, mut for those who carry the minor variant, in our cohort those were all heterozygous). Ileal CD patients show reduced expression levels of HD5 as compared to controls which are the lowest in carriers of the minor C- allele. The stratification according to the presence of the functional LRP6 mutation seems to have, as expected, no effect on LRP6 mRNA level. Both for HD5 and LRP6, there was no difference between exclusive ileal (L1, n=3) and ileocolonic CD (L3, n=6) in the carriers of the minor variant.

3. LRP6 mRNA expression is reduced in ileal CD

We found no difference in the transcriptional expression of LRP6 between controls who were subgrouped according to the genotype as well as between the subgrouped ileal CD patients but found generally diminished LRP6 mRNA in ileal CD. This genotype independently impaired expression was furthermore also seen in 6 patients with uninflamed mucosa at the time of biopsy sampling. We also found the same lack of an effect on HD5 mRNA level in ileal CD patients after stratification according to the inflammatory status (1 out of 6 was a C-allele carrier in the uninflamed group as compared to 5 out of 25 in the inflamed group). We saw a trend for higher levels of HD5 in uninflamed L3 patients as compared to uninflamed exclusive ileal CD (L1) and therefore checked for this in all samples. A similar effect became evident in the overall analysis of stratified small intestinal CD patients, which was also seen for LRP6 but not significant in either case.

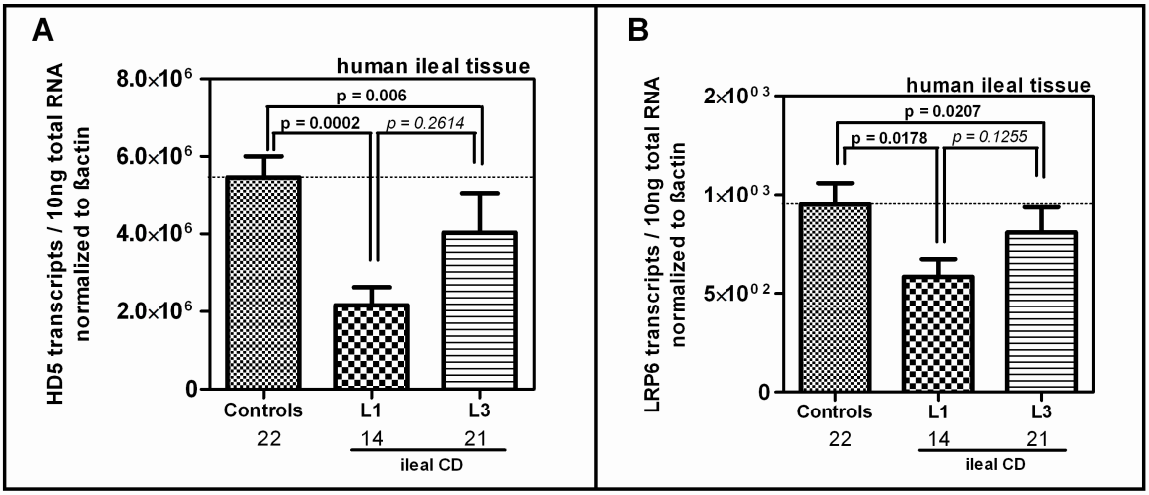

Figure 3.7: **LRP6 and HD5 mRNA in ileal CD.** Expression levels of both HD5 and LRP6 seem to be marginally higher in ileocolonic CD (L3) as compared to exclusive ileal CD (L1), which did not achieve significance in the overall samples. Most importantly though, both ileal CD subgroups show reduced expression of both factors compared to healthy controls.

Since we could not detect an effect of the mutation on HD5 mRNA levels in controls, the reduced transcriptional expression of LRP6 might be an additional factor which contributes to the especially low levels seen in LRP6 mutated patients. We performed Spearman rank analysis in the samples and found that mRNA level of LRP6 correlated with the Paneth cell antimicrobial HD5 in controls as well as in all individuals who were homozygous for the more common wildtype (WT) variant of the SNP.

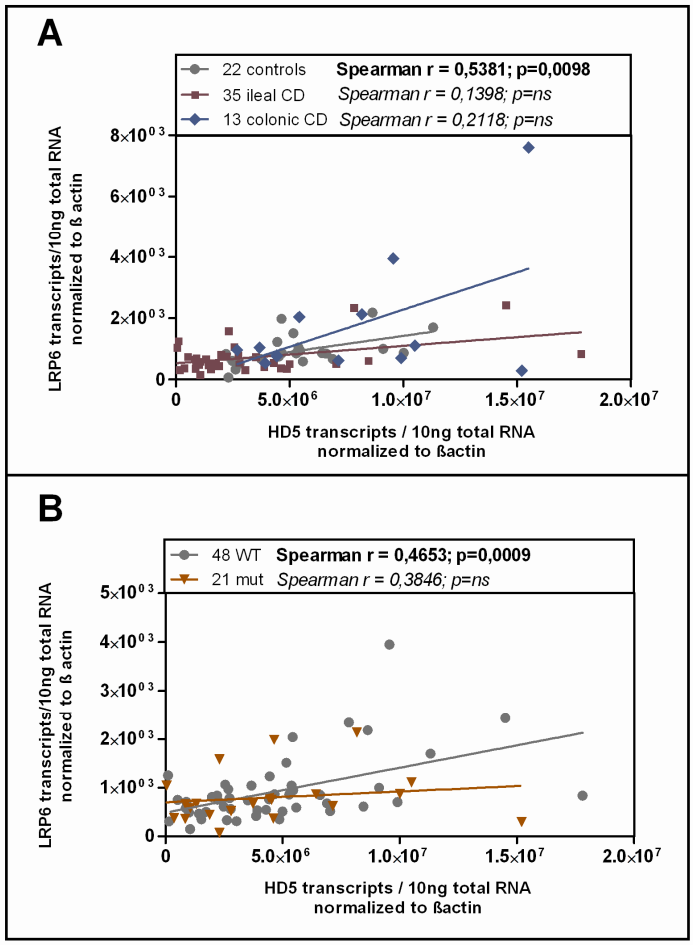

Figure 3.8: **Correlation of HD5 and LRP6.** Linear regression and Spearman rank analysis were used on all samples from the mRNA analysis. The samples were grouped according to their disease state (A) as well as in all samples according to the LRP6 genotype (B). Interestingly the two factors exhibit a correlating pattern in controls (independently to the genotype) as well as in all samples with a wildtype but not in mutated individuals.

Taken together, both, the mutation, as well as the diminished expression level of LRP6, potentially in an additive fashion, might influence the transcription levels of HD5 in ileal CD patients. Since HD6, the second most abundant Paneth cell product is also reduced in ileal CD (Wehkamp et al., 2005b), we additionally analyzed its expression according to the LRP6 genotype in our patients. It is known that both Paneth cell α-defensins are regulated by the Wnt pathway (Wehkamp et al., 2007) so we expected a similar effect. As hypothesized, the two factors showed a correlating pattern in wildtype as well as in mutated individuals and HD6 exhibited the same dependence on the LRP6 genotype which was seen for HD5 in ileal CD. We also measured Lysozyme,

another antimicrobial found in Paneth cells, which is not decreased in ileal CD and also not known to be dependent on canonical Wnt. There was, as expected, no change of Lysozyme mRNA levels in ileal CD carriers of the minor LRP6 SNP compared to the wildtype ileal CD subgroup.

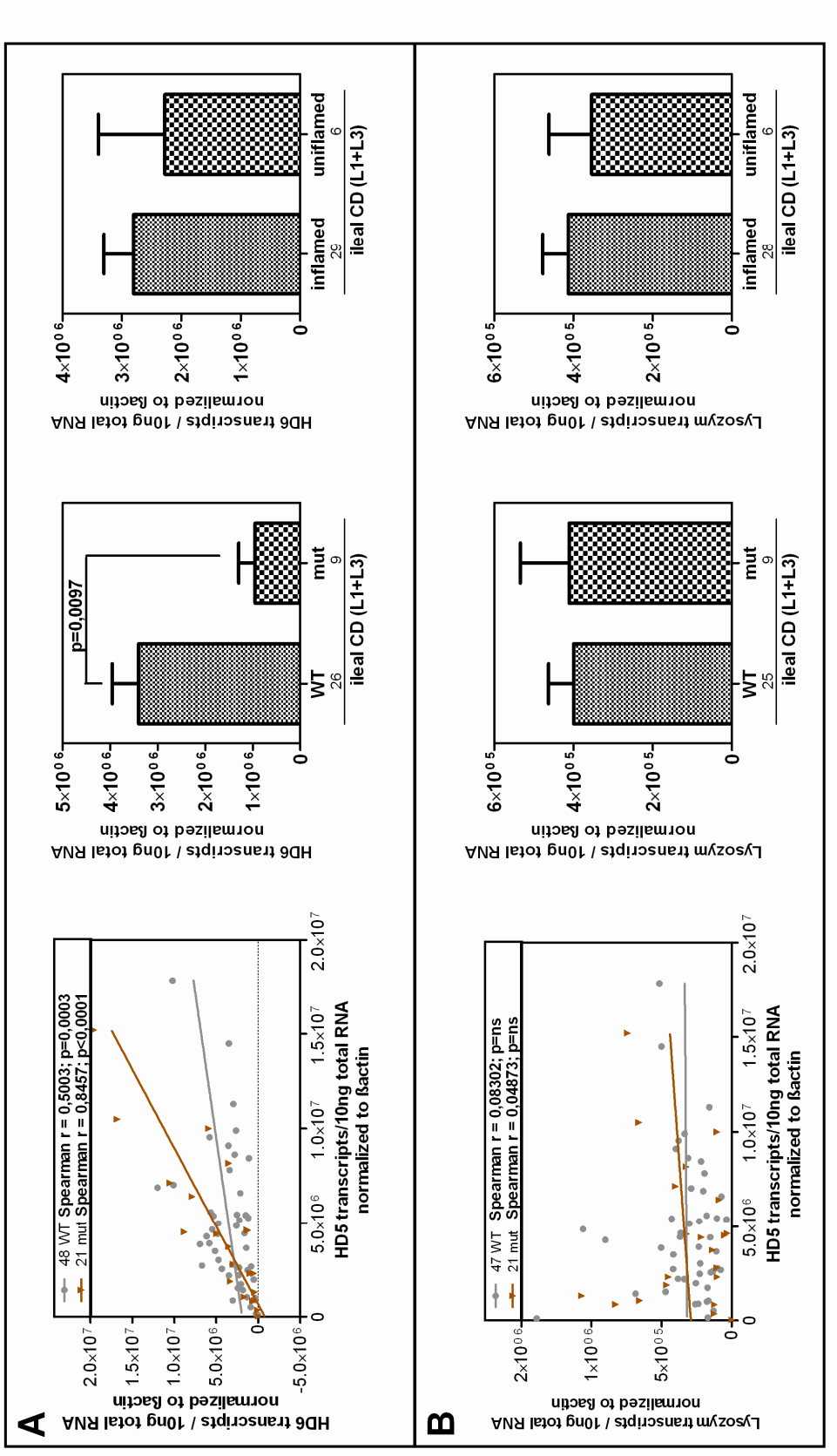

Figure 3.9 **HD6 is also further reduced in patients who carry the minor LRP6 variant, which is not seen for Lysozyme.** Linear regression and Spearman rank analysis were used on all samples from the mRNA analysis according to the LRP6 genotype. (A) The two Paneth cell α-defensins correlate in both subgroups which fits the also found genotype dependent decrease of HD6 in ileal CD patients. This, as well as the independence from inflammation matches the pattern seen for HD_5. (B) Lysozyme on the other hand doesn not correlate with HD_5 and seems to be uninfluenced by the LRP6 genotype in ileal CD.

Discussion

Key results

In order to complement the previously described involvement of *TCF7L2* (Wehkamp et al., 2007; Koslowski et al., 2010) as a disturbed β-catenin dependent Wnt pathway factor, we wanted to investigate a further crucial component of the signaling cascade in ileal Crohn's Disease. With the herein presented target gene approach results, we can now report an association of a rare coding SNP variant (C-allele, rs2302685, Ile1062Val) in the Wnt co- receptor *LRP6* with early-onset ileal CD. An additional genotype independent reduction of LRP6 mRNA level in ileal CD biopsy tissue was also found as a second impairment in patients. From studies in the human setting it is known that the presence of the associated minor coding variant results in generally diminished signaling activity of LRP6 (De Ferrari et al. 2007). Since from animal studies it is also known that a general intestinal blockage of the Co-receptor leads to epithelial degeneration and eventual inflammation (Hoffman et al., 2004), we hypothesized on a functional relevance of the coding SNP in the disease setting. We therefore wanted to analyze its role in ileal CD and found significantly further reduced HD5 and also HD6 expression levels in ileal biopsy isolates from patients who carried the minor C-allele. Since the variant showed an additional weaker association with penetrating disease behavior, the further reduction of Paneth cell α-defensins might be of clinical relevance in adult patients even though the causative LRP6 variant was not associated within adult ileal CD in general.

Limitations of the study

Even though the association of the coding rare variant of rs2302685 with early onset ileal CD was found in three different DNA cohorts, the overall number in the affected subgroup was quite small as unfortunately only a limited fraction (~12%) of the studied ileal CD patients shared this phenotype. Other limitations of the study lie in the lack of homozygous C-allele carriers in the mRNA sample study and that it was carried out using only adult patients. Both are due to restricted biopsy material resources. A

potentially even stronger effect in homozygous C-allele carriers or a cause-effect-relationship in pediatric ileal CD remains therefore to be investigated.

Open questions and potential future investigations

It was surprising, that exclusive colonic CD (L2) patients seemed to have quite high LRP6 mRNA expression compared to controls and also fairly high levels of HD5 and LRP6 in the C-allele carrier subgroup compared to those homozygous for the more common ancestral allele. Both differences were not statistically significant and the effects might likely be due to the low numbers of analyzed patients within these groups. Nonetheless it would be interesting to further investigate both factors in exclusive colonic CD. The high levels of HD5 might help to explain the healthy small intestinal epithelia in the otherwise affected intestine of L2 patients. The associated mutation only affects a small fraction of ileal CD patients who nonetheless in general exhibit diminished HD5 expression (Wehkamp et al, 2005b; Bevins et al., 2009, Elphik et al., 2005; Mahida et al. 2005). It is therefore obvious that additional mechanisms influence deficient Paneth cell innate immunity in ileal CD patients. Since control individuals who feature the C-allele heterozygously also showed almost normal mRNA levels of HD5, it is also clear, that such mechanisms might additionally explain why the effect of the mutation is specific to ileal CD. Patients who carry the minor variant could be prone to an early symptom development only in combination with other underlying disturbances which could be the same mechanisms that explain diminished HD5 expression in ileal CD in general. One potential additive mechanism might be the diminished transcriptional expression of LRP6, which was seen independently from the patient's genotype. It might represent a second and maybe more general cause of low HD5 and complement the previously described diminished expression of TCF7L2. In order to generate a better understanding of ileal Crohn's Disease pathogenesis it will be crucial to analyze the putative additive function of the reported impairments in canonical Wnt. Such studies as well as other investigations on Paneth cell regulating factors will have to shed light on the exact circumstances and especially on the subphenotype specifics in ileal CD.

As mentioned, the associated minor coding rs2302685 variant has previously been shown to inhibit the receptors capacity as it decreased Wnt promoted β- catenin stability. The same study could associate the more common, normally functioning ancestral allele of the SNP with susceptibility towards late onset Alzheimer's disease (De Ferrari et al., 2007). Weather this condition might negatively correlate with early onset ileal CD will be hard to elucidate. Studying a potential impair in the regenerative potential of intestinal epithelia in patients though, might be a more promising approach. In adult mice, this was already partly performed by adenoviral expression of the secreted Wnt antagonist Dickkopf-1 (Dkk1). Dkk1 specifically inhibits canonical Wnt pathway through interaction and interference with LRP6. As mentioned in the introduction, this approach revealed rapid inhibition of small intestinal and colonic epithelial regeneration, loss of proliferative crypts, and eventual inflammation and architectural degeneration (Hoffman et al., 2004). It is imaginable that such complications might, to a reduced extent, also occur in carriers of the rare rs2302685 allele. Intestinal barrier effectiveness of corresponding patients would then additionally be compromised by disturbances in cell renewal and/or epithelial healing. Certainly future research is required but if the concept proves relevant, new therapy approaches and drug targets might become conceivable and needed.

LRP6 impairments in small intestinal CD and the importance of genotyping

Ileal CD patients who were affected by both, the functional minor variant and reduced expression of LRP6 exhibited the lowest HD5 levels in the studied sample set. Patients with diminished but wild-type LRP6 also showed an mRNA reduction but this was not significant when compared to healthy controls with normal expression levels of LRP6 when they were featuring the rare coding C-allele. Taken together, both, the mutation as well as the diminished expression of LRP6 might influence the transcribed levels of *HD5*. It is quite evident that such previously unknown effects of a coding SNP variant could provide a bias in mRNA studies which investigate HD5 and also HD6. Since it can't be excluded that various other variants with similar influences have not been detected yet, the need for adequately large cohorts in transcriptom studies seems

rather high. Since we cannot exclude such an unknown bias in our small set, the potential influence of the LRP6 coding variant needs further confirmation in a larger cohort and on a functional level. Nonetheless, besides providing a potential diagnostic marker for the identification of high risk individuals, the functional *LRP6* SNP should, similarly to the variants in *NOD2*, be taken in consideration when HD5 and HD6 expression levels are analyzed.

As previously mentioned, studies on *LRP6* genetic variances have already been done in the context of variability in bone mass density (Sims et al., 2008) and bone disorders (van Meurs et al., 2008) amongst others. Complications affecting the bone are frequent in IBD. The newly reported genetic association of *LRP6* might have an effect in worsening the occurrence of such extraintestinal manifestations. Future studies would need to analyze weather patients with the mutation require additional monitoring in order to avoid detrimental effects of certain anti-inflammatory medications.

Disease mechanisms in pediatric CD and the role of LRP6

The decrease of TCF7L2 in ileal CD, which our group reported, could recently be confirmed in the pediatric form of the disorder (Perminow et al., 2010; Zilbauer et al., 2010). Since diminished TCF7L2 and HD5 in affected children match the defects seen in adult patients, the mechanism doesn't help to discriminate between early and late disease onset. It rather emphasizes a common character of the defect. Interestingly, most previously in genetic analyses identified Crohn's disease loci (Scherr et al., 2009; Imielinski et al. 2009) are also common to both early and later onset. Exclusive early onset disease variants are extremely rare and none are known for the ileal version specifically. Recently an IL6 promoter SNP was associated with early onset CD in general but confined solely to male patients (Sagiv-Friedgut et al. 2010). Five novel genomic regions were additionally associated with general early-onset IBD in a genome wide association study (Imielinski et al. 2009). The identification of rs2302685

as an early onset and also penetrating ileal CD risk variant potentially provides a first specific diagnostic marker for respective risk individuals.

Wnt signaling as a potential therapeutic target in small intestinal CD

In intestinal disorders it is mainly the consequences of increased Wnt signaling which have been taken in account, as an over- activation of the pathway is present in the majority of colorectal cancers (de Ridder et al., 2007; Segditsas and Tomlinson, 2006). Tinkering with the idea of Wnt pathway manipulating to enhance antimicrobial defense and regeneration in ileal CD is therefore challenging. Nonetheless new strategies could alternatively target steps in Wnt besides controlling inflammatory pathways as it is currently the mainstay of therapy. Factors which have already been in discussion as regenerative drugs form a class of canonical Wnt boosting intestinotrophic mitogenes, the already mentioned R-Spondins. They have been shown to specifically bolster LRP6 capacity (Kim et al., 2008) and proved effective in ameliorating tissue destruction in different mouse models (Zhao et al., 2007; Zhao et al., 2009). Interestingly, a recent paper dealing with rectally administered recombinant human hepatocyte growth factor (HGF) in rat experimental colitis followed a similar idea in the colonic form of IBD (Setoyama et al.2011). The recombinant HGF accelerated the mucosal repair; whereas serum levels of the locally administered morphogene were undetectable. Since the proliferation of uninjured mucosa is not negatively affected, the authors concluded that HGF might provide a potential new therapy approach in colonic IBD. Another recent study on intestinal mucosal repair dealt with a similar hypothesis. It was reported that the depletion of the LRP6 antagonist Dkk1 promoted a strong proliferative response and wound repair after chemically induced colitis in mice (Koch et al., 2011). During colitis, mice with reduced Dkk1 expression, which indeed recovered significantly faster, also exhibited crypt irregularities and epithelial hyperproliferation in the large intestine. An activation of the Wnt pathway in this compartment might therefore be of a short term benefit in restoring epithelial integrity but has the potential to promote malignant hyperproliferation. In small intestinal CD, a location specific application of LRP6

activating factors, like R-Spondin proteins (Binnerts et al., 2007), could not only support epithelial regeneration and Paneth cell function but also avoid deregulation of the pathway in the large bowel. Primary small bowel cancers are rare. Even though the small intestine constitutes over 75% of the length and 90% of the mucosal alimentary tract surface, it only accounts for 1% of gastrointestinal cancers (~ only 6000 cases/year in the United States)(Gore, 1997; Zouhairi et al., 2008). An involvement of Wnt activation in small intestinal cancer is comparably scarcely discussed. Stabilization of β-catenin occurs in some small intestinal carcinomas (Breuhahn et al., 2008) but mutations of the APC gene or β- catenin point mutations, which are frequently activating canonical Wnt in CRCs have not been found (Wheeler et al., 2002). This suggests that small intestinal carcinogenesis may follow different genetic pathways then CRCs. One explanation might be a potential redirection of overactive β- catenin signaling towards increased Paneth Cell differentiation in this tissue. Corresponding effects are seen in mice with acute Wnt activation (Andreu et al., 2005) and similar ideas are already taken into considerations in other tissue settings like for example in skin (Chien et al., 2009). In some ileal CD patients, the decrease of and the functional mutation in LRP6, entailed defects in antimicrobial barrier function and the Paneth cell dependence on Wnt might provide one of the first and a relatively ideal target for canonical Wnt pathway activating drugs.

Generalisability

Finally it is important to acknowledge the context of the LRP6 association as it provides further evidence for the importance of the Paneth cell in the development of small intestinal CD. Multiple genetic CD variants have already been specifically associated with the small intestinal disease subgroup. Most of the involved genes are important in the specialized cell's biology and its antimicrobial defense system which will be discussed in detail in chapter 3.4.2.

Own contribution

My contributions to this work were in conceiving the study, the candidate gene SNP selection, the Sequenom assay design, the genotyping of the 3 European DNA cohorts and the samples from Stuttgart, which were used in the mRNA analysis. I furthermore selected the samples for the mRNA analysis and measured HD5, HD6, Lysozyme, β-actin and LRP6 mRNA expression in most of the Stuttgart cohort, analyzed all data and prepared all graphs.

Other contributions

W. Reinisch, A. Teml, P. Rutgeerts, S. Vermeire, R. Cooney, D. Jewell who are already mentioned in Koslowski et al., 2010, provided the DNA and all information on the DNA population cohorts. E. Schaeffeler provided support in the design and evaluating of genotyping assays. I. Kübler and G. Wang were involved in the candidate gene selection and in discussion. J. Beisner supervised the mRNA quality control and was, together with Z. Teltschik involved in discussion, assemblance of the mRNA study and the mRNA measurement of some of the Stuttgart samples. E. F. Stange was involved in discussion of the data. J. Wehkamp was decisively involved in designing the study and in data discussion.

3.3 Influence of standard treatment on ileal and colonic antimicrobial Defensin expression in active Crohn's disease

I. Kübler, M. J. Koslowski, M. Gersemann, K. Fellermann, J. Beisner, S. Becker, K. Rothfuss, K. R. Herrlinger, E. F. Stange, J. Wehkamp

Synopsis

Background

Patients with Crohn's disease exhibit diminished bacterial killing activity at their intestinal mucosa due to location specific defects in the production of antimicrobial peptides as described in the introduction. Even though no causal therapies are available for the different forms of CD (Stange et al., 2006; Travis et al., 2006), medications like corticosteroids, thiopurines, methotrexate or TNF-antibodies can dampen disease symptoms due their inflammation repressing properties (Travis et al., 2006). To investigate potential effects of such standard medication on intestinal antimicrobials, we selected 75 patients with documented current treatment who underwent diagnostic colonoscopy due to a clinical relapse. The age distribution was typical as was the balanced gender ratio. Mucosal samples were stratified according to ileal (all either L1 or L3) or sigmoid biopsy site (all either L2 or L3) as well as the concurrent use of corticosteroids, azathioprine and 5-Asa. Finally we combined the patient samples into groups with low (<1000 copies/10ng total RNA) or high (1000 – 1000 000 copies/10ng total RNA) IL8 expression, indicating moderate to severe inflammation.

Main results

Conform to previously published data, the major defensin expressed in the terminal ileum was HD5, followed by HD6 and at a hundredfold lower level HBD1 and HBD2, whereas the cathelecidin LL37 and HBD3 exhibited the lowest mRNA levels. The major β-defensins expressed in colonic mucosa were HBD1 and the inducible HBD2, followed by HBD3 with a matching expression pattern and at the lowest level LL37. We found no significant changes in any of the treatment groups. In agreement with previous studies

(Wehkamp et al., 2004a; Wehkamp et al., 2005a) supporting a primary decrease of the factors, the degree of inflammation did not affect HD5 and HD6 mRNA levels in the analyzed samples. Based on results from a previous contradicting Australian study, differences according to the severity of inflammation, respectively the tissue damage would be expected. We did not find such differences and also did not see a negative correlation between HD5 or HD6 and inflammatory cytokine level. A reason for the unequal interpretations might be differences in study design or an unequal and heterogenic genetic background of the included individuals. The data presented here, further support that the decrease of Paneth cell α-defensins is no secondary effect due to epithelial loss. Expression levels of all measured inflammatory cytokines though were highly matching and on a weaker base also correlated with the anti-inflammatory IL10. As not surprising for HBD2, we found a significant increase in the high inflammation group and a correlation with inflammatory cytokines in both ileal and colonic sample sets, which was also observed for LL37.

Own contribution

My contributions to this work were the measurement of ileal and colonic LL37 and HBD1 mRNA expression, the subgrouping and data analysis of all factors according to inflammation severity, analyzing the percentile distribution of the measured antimicrobials, calculation of correlation coefficients, preparation of all graphs and in large parts in the discussion of results and in writing of the manuscript

Other contributions

I. Kübler selected patient samples and coordinated their preparation (mRNA isolation, cDNA synthesis), measured expression levels of factors other than HBD1 and LL37 and participated in discussion and writing of the manuscript. M. Gersemann and S. Becker were involved in the design of assays for the measurement of cytokines. K. Fellermann was previously involved in the design of RT-PCR assays for the measurement of defensins. M. Gersemann, K. Fellermann, K. Rothfuss, K.R. Herrlinger were involved in recruiting patients and phenotyping of the cohort. J. Beisner supervised the mRNA

quality control and was involved in discussion. J. Wehkamp and E. F. Stange conceived the study and were involved in discussion and manuscript writing

Alimentary Pharmacology & Therapeutics

Influence of standard treatment on ileal and colonic antimicrobial defensin expression in active Crohn's disease

I. KÜBLER*, M. J. KOSLOWSKI†, M. GERSEMANN*, K. FELLERMANN‡, J. BEISNER†, S. BECKER†, K. ROTHFUSS*, K. R. HERRLINGER*, E. F. STANGE* & J. WEHKAMP*,†

*Robert-Bosch-Hospital, Stuttgart, Germany; †Dr. Margarete-Fischer-Bosch Institute of Clinical Pharmacology and University of Tübingen, Stuttgart, Germany; ‡Department of Internal Medicine I, Division of Gastroenterology, University of Schleswig-Holstein, Lübeck, Germany

Correspondence to:
Dr. med. J. Wehkamp, Department of Gastroenterology, Dr. Margarete-Fischer-Bosch Institute of Clinical Pharmacology, Robert-Bosch-Hospital, Auerbachstr. 112, 70376 Stuttgart, Germany.
E-mail:
jan.wehkamp@ikp-stuttgart.de

Publication data
Submitted 8 April 2009
First decision 16 April 2009
Resubmitted 31 May 2009
Accepted 14 June 2009
Epub Accepted Article 22 June 2009

SUMMARY

Background
Crohn's Disease (CD), a chronic intestinal inflammation, is currently tre ted primarily by therapeutics which are directed against inflammato responses. Recent findings though suggest a central role of the inna immune barrier in the pathophysiology. Important factors providing th barrier are antimicrobial peptides like the α- and β-defensins. Little known about *in vivo* effects of common drugs on their expression.

Aim
To analyse the influence of corticosteroids, azathioprine and aminos licylate treatment on ileal and colonic antimicrobial peptides in acti CD and also assess the role of inflammation.

Methods
We measured the expression of antimicrobial peptides and pro-inflan matory cytokines in 75 patients with active CD.

Results
Ileal and colonic α- and β-defensins as well as LL37 remained una fected by corticosteroids, azathioprine or aminosalicylate treatmer Additionally, we did not observe a negative coherency between Pane cell α-defensins and any measured cytokines. HBD2 and LL37 unli HBD1 levels were linked to inflammatory cytokines and increased highly inflamed samples.

Conclusions
Current oral drug treatment seems to have no major effect on t expression of antimicrobial peptides. In contrast to HBD2 and LL3 ileal levels of HD5 and HD6 and colonic HBD1 level are independent current inflammation. Innovative drugs should aim to strengthen pr tective innate immunity.

doi:10.1111/j.1365-2036.2009.04070.x

INTRODUCTION

Crohn's disease (CD) is characterized by a chronic and severe inflammation of the intestine with diarrhoea, deep ulcerations and – in some cases – multiple fistulas.[1] Both genetic and environmental factors were shown to contribute to disease risk.[2] The leadoff but aged pathogenic disease concept was based on the idea of a 'dysregulation' in adaptive immunity.[3, 4] Disease symptoms caused by an 'over-reactive' immune response at the mucosa may be successfully dampened by corticosteroids, thiopurines, methotrexate or tumour necrosis factor (TNF)-antibodies.[5] Therefore, immunoregulatory agents remain the mainstay of conservative therapy in CD. However, the achievement of both induction and long-term maintenance of remission are still unsatisfactory.[6]

More than 10 years ago, it became evident that activated T-cells as well as antibodies in CD targeted bacterial flora.[7–9] Inflammatory affected host tissue would therefore mostly count for collateral damage, rather then being the prime target of misdirected adaptive immune cells. On the basis of consecutive research, we developed an alternative but not mutually exclusive hypothesis, focusing on impaired barrier function with a secondary inflammatory response.[10] One of its basic principles assumes that a healthy gut epithelium keeps the normal flora at a safe distance. This is achieved through a protective mucus layer containing defensins, cathelicidins and other antimicrobials as a first line of defence.[11] The barrier thereby not only balances the commensal – host relationship at the mucosa, but also provides an effective protection against ingested pathogens.[12] This barrier results in the observation that infections, stimulation of the immune system and subsequent inflammation are the exception rather then the rule in the healthy gastrointestinal tract. Crohn's disease, however, was found to be associated with mucosal adherent bacteria.[13–15] One likely but not exclusive explanation for this characteristic are localization-specific deficiencies in different antimicrobial peptides at the mucosal surface.[16] In ileal CD patients, diminished Paneth cell α-defensin expression leads to reduced antibacterial killing activity of the mucosa.[17] These α-defensins, the human alpha-defensin-5 (HD5) and human alpha-defensin-6 (HD6), are the most abundant Paneth cell products and the prominent antimicrobial peptides in the ileal mucosa.[18, 19] Mechanisms underlying their decrease are represented by a genetic background with variations in several genes: NOD2,[20, 21] ATG16L1[22] and TCF4.[23] Those genes, all linked with a small intestinal involvement of CD are important players in Paneth cell biology and pinpoint to a primary and paramount role of this cell in the disease process.

In contrast, colonic CD is characterized by an attenuated induction of inducible β-defensins.[24–26] This defect in inducing these protective host factors is also explained by genetics, respectively by low gene copy numbers of human-beta-defensin-2 (HBD2).[27] HBD2 has been shown to be induced not only by different pathogens[28, 29] but also specifically by probiotic bacteria.[30–32] In this process, probiotics are probably killed by the antimicrobial peptide they induce, but leave the host with an enhanced protective barrier.[30] A defect in β-defensin induction might partly explain why probiotic treatment seems to have promising effects in pouchitis and the maintenance of remission in ulcerative colitis, but no benefit in CD.[33]

In the present study, we assessed the impact of standard medications in a translational disease patient study on the expression of various important defensins. The findings were also related to the degree of inflammation as measured by cytokine expression.

PATIENTS

A total of 75 Crohn's disease patients were included in the study and their current treatment was documented. Patients underwent diagnostic colonoscopy because of a clinical relapse. The study was approved by the ethics committee of the University of Tübingen. After informed consent, biopsies were collected from macroscopically inflamed Crohn's disease tissue of the small (n = 53) and large intestine (n = 44) and immediately shock-frozen into liquid nitrogen.

METHODS

RNA isolation and cDNA synthesis were performed as described before.[18] Levels of mRNA expression from human alpha-defensin-5 HD5 (DEFA5), human alpha-defensin-6 HD6 (DEFA6), human beta-defensin-1 HBD1 (DEFB1), human beta-defensin-2 HBD2 (DEFB4), human beta-defensin-3 HBD3 (DEFB103A), cathelicidin antimicrobial peptide LL37 (CAMP), interleukin-1β (IL1B), IL6, IL8, IL10, intercellular adhesion molecule 1 (ICAM1) and TNF (TNF superfamily, member 2, TNFα) were quantified by real-time RT-PCR using the LightCycler 2.0 and 480 (Roche Diagnostics GmbH, Roche

Applied Science, Mannheim, Germany). Primers for HD5, HD6, IL-8, TNFα;[21] HBD1, HBD2,[34] HBD3,[27] LL37[35] and IL1B[36] were previously published. Primer IL6 sense: 5′ AAT CAT CAC TGG TCT TTT GGAG 3′; antisense: 5′ GCA TTT GTG GTT GGG TCA 3′; Primer IL10 sense: 5′ AAG CCT GAC CAC GCT TTC TA 3′; antisense: 5′ ATG AAG TGG TTG GGG AAT GA 3′; Primer ICAM1 sense: 5′ TAG CAG CCG CAG TCA TAA 3′; antisense: 5′ CGG GAT AGG TTC AGG GAG 3′. Absolute quantification was determined by external standards.[18]

We stratified the mucosal samples according to ileal or sigmoid biopsy site as well as the concurrent use of corticosteroids, azathioprine and 5-Asa. Patient samples were combined into groups with low (<1000 copies/10 ng total RNA) or high (1000–1 000 000 copies/10 ng total RNA) IL8 expression, indicating moderate-to-severe inflammation. The Mann–Whitney *U*-test was performed for comparison of grouped data. *P*-values of <0.05 were considered statistically significant.

RESULTS

Patient characteristics

The patient characteristics are given in Table 1. The age distribution was typical for this disease as was the balanced gender ratio. Patients with ileal biopsies all had localization L1 or L3 and those with sigmoid biopsies exclusively belonged to Vienna categories L2 or L3.[37] Disease behaviour distribution in both groups was similar. The number of patients on or off steroids was rather balanced in this cohort, whereas the numbers of patients on azathioprine or 5-ASA were smaller. Very few patients additionally received the nutritional supplement zinc (three in the ileal and five in the colonic group), two patients with ileal biopsies taken and five with colonic biopsies received antibiotics and in both groups, one patient was additionally treated with anti-TNF antibodies.

Table 1. Characteristics of patient cohort

	Ileum	Sigmoid
Age (median ± s.d.)	36 ± 13	37 ± 15
Gender ratio (m/f)	25/28	13/31
Vienna A1/A2	47/6	34/10
Vienna L1/L2/L3	25/0/28	0/19/25
Vienna B1/B2/B3/unknown	19/17/15/2	21/8/13/2
Corticosteroids (on/off)	23/30	23/21
Azathioprine (on/off)	10/43	9/35
Aminosalicylate (on/off)	14/39	15/29

s.d., standard deviation; m, male; f, female; A, age of onset; L, localization; B, behaviour; according to Vienna classification.[37] Data shown in absolutes numbers.

Ileal defensin expression

The major defensin expressed in the terminal ileum was HD5, followed by HD6 and at a hundredfold lower level, by HBD1 and HBD2, whereas the cathelecidin LL37 exhibited the lowest mRNA level. None of the studied therapies significantly affected the expression of both α- and β-defensins, as well as LL37 (Figure 1 and Table 2). As expected, for ileal HD5 and HD6 expression, a significant ($P < 0.0001$) and strong (Spearman $r = 0.9486$) correlation has been found (Figure 2). Furthermore, supporting the hypothesis of an inflammation independent and primary role of Paneth cell α-defensins in ileal CD, no negative coherency between either HD5 or HD6 mRNA level and inflammatory cytokine expression was observed (Table 3, Figure 2). Grouping the samples according to IL8 expression levels which correlates with the histological grade of inflammation,[38] we again found no difference in Paneth cell α-defensin expression. The expression level of all measured inflammatory cyctokines was highly matching and on a weaker base also correlated with the anti-inflammatory IL10 (Table 3). Ileal expression of HBD3 was minimal (Table 2). Not surprisingly, for HBD2, we found a significant increase in the high inflammation group (data not shown), which could also be observed for LL37 mRNA level (Figure 5). MRNA level of both antimicrobial peptides correlated with the expression of inflammatory cytokines (Table 3).

Colonic defensin expression

The major β-defensins expressed in colonic mucosa were HBD1 and the inducible HBD2, followed by HBD3 and at the lowest level, LL37 (Figures 3 and 5, Table 4). The two inducible HBDs exhibited matching expression patterns (Spearman $r = 0.7484$; $P < 0.0001$). HBD2 mRNA levels also correlated with those of the measured inflammatory cytokines (Table 5, Figure 4). The strongest effect was seen with IL1B (Spearman $r = 0.5838$; $P < 0.0001$). HBD1 however, exhibited mRNA levels independent of HBD2 and

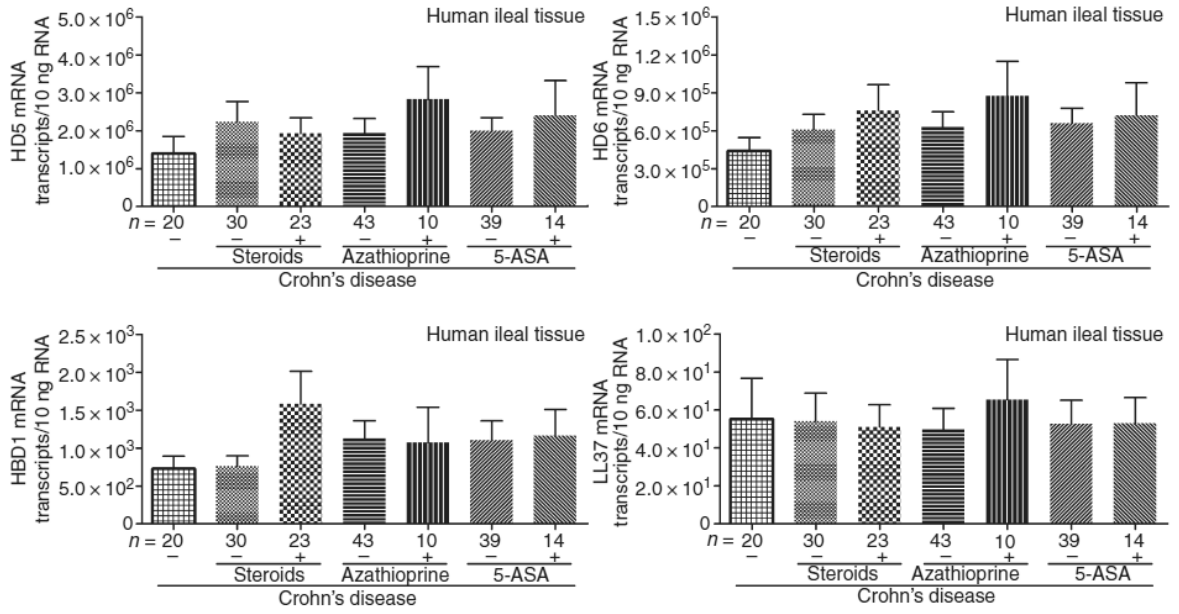

Figure 1. Current treatment and the expression of ileal defensins and LL37 in active Crohn's Disease. In Crohn's disease patients, the ileal expression of antimicrobial Paneth cell α-defensins is not significantly affected by the studied therapies (corticosteroids, azathioprine and aminosalicylates) (upper panel). Similarly, the constitutively expressed β-defensin HBD1 as well as LL37 seems unchanged (lower panel).

inflammatory cytokines (Table 5, Figure 4). Consistent with these results, we found an increase in HBD2 in the high inflammation group, whereas HBD1 remained unchanged (Figure 4). Similar to HBD2, LL37 showed an increased expression after stratifying the samples according to inflammation severity and analogous mRNA levels in comparison with cytokine expression (Figure 5 and Table 5). There were no significant differences in the treatment groups comparing HBD2, HBD3 and LL37 expression levels in the colonic mucosa. We saw a HBD1 decrease in the 5-ASA 'on' compared to the 'off' group, which did not hold up after *P*-value adjustment according to multiple testing (Figure 3). In conclusion, none of the treatments seemed to have any paramount effect on antimicrobial peptide expression.

DISCUSSION

The present study is the first which systematically assessed the effect of standard treatments in Crohn's disease on the expression of important barrier peptides in affected patient mucosa. It should be noted that all patients had an indication for the endoscopy because of a clinical relapse. As a consequence, none of the patients were in clinical remission and, indeed, none in endoscopic remission. We stratified the samples according to the severity of inflammation which was determined by IL8 mRNA levels.[17] We found a strong link between IL8 and all other proinflammatory cytokines in either tissue, supporting its beneficial use as a marker.

Patients with ileal localization of CD did not show significant differences in mRNA expression of antimicrobial peptides under standard medication including corticosteroids, azathioprine or aminosalicylates. The lack of an effect on α-defensins was not surprising. These peptides are constitutively expressed and appear to be under the control of different factors including the intracellular receptor nucleotide binding oligomerization domain NOD2. The detailed mechanisms behind the link between Paneth cell α-defensins and NOD2 are not clearly understood, but supported by investigations in NOD2 knockout mice[39] as well as by translational studies in European[34, 40] and US patients.[41, 42] In addition, Paneth cells are adversely affected by

Table 2. Expression levels of antimicrobial peptides and cytokines: mRNA transcripts/10 ng RNA in human ileal tissue

Products	Steroids				Azathioprine				5-ASA			
Medication	–		+		–		+		–		+	
n	30		23		43		10		39		14	
HBD1	726.7 (±166.4)	762.1 (±136.8)	1585 (±426.2)	1129 (±232.6)	1076 (±463.1)	1104 (±253.5)	1163 (±345.6)					
HBD2	1766 (±975.7)	1966 (±875.9)	912.4 (±254.5)	1745 (±620.7)	494.3 (±265.1)	1304 (±517.4)	2080 (±1309)					
HBD3	1558 (±1234)	1077 (±826)	187.8 (±135.7)	849.8 (±579.9)	7.64 (±3.323)	909.5 (±639)	81.81 (±74.58)					
HD5	1 403 000 (±442 410)	2 240 000 (±532 497)	1 933 000 (±406 767)	1 938 000 (±378 721)	2 831 000 (±857 263)	1 997 000 (±347 582)	2 412 000 (±910 334)					
HD6	440 552 (±106 481)	610 644 (±118 496)	761 554 (±201 771)	629 730 (±119 640)	875 667 (±274 286)	659 779 (±118 727)	721 689 (±258 723)					
LL37	55.35 (±21.47)	54.23 (±14.78)	51.24 (±11.67)	49.99 (±10.96)	65.59 (±21.14)	52.86 (±12.35)	53.15 (±13.49)					
IL8	36 741 (±33 357)	27 005 (±22 215)	10 128 (±4830)	23 664 (±15 629)	2556 (±1010)	24 734 (±17 232)	5606 (±2370)					
IL6	4961 (±3452)	3317 (±2202)	1295 (±926.8)	2967 (±1604)	169.5 (±46.87)	3272 (±1811)	353.9 (±171.1)					
IL10	1535 (±441.0)	1267 (±313.5)	844.7 (±181.6)	1189 (±235.7)	632 (±136.5)	1217 (±251.4)	713.5 (±212)					
IL1B mean (±S.E.)	67 464 (±34 513)	53 789 (±23 517)	16 454 (±6066)	44 725 (±16 730)	6892 (±3066)	44 401 (±18 218)	18 605 (±10274)					
TNFα mean (±S.E.)	1764 (±672.7)	1447 (±444.1)	609.7 (±110.7)	1171 (±318.1)	644.5 (±160.7)	1192 (±340.7)	737.5 (±268.2)					
ICAM1	5869 (±2688)	5322 (±1841)	3314 (±818.3)	4971 (±1371)	2710 (±678.3)	5006 (±1438)	2903 (±1125)					

Shown are the means ± the standard errors.

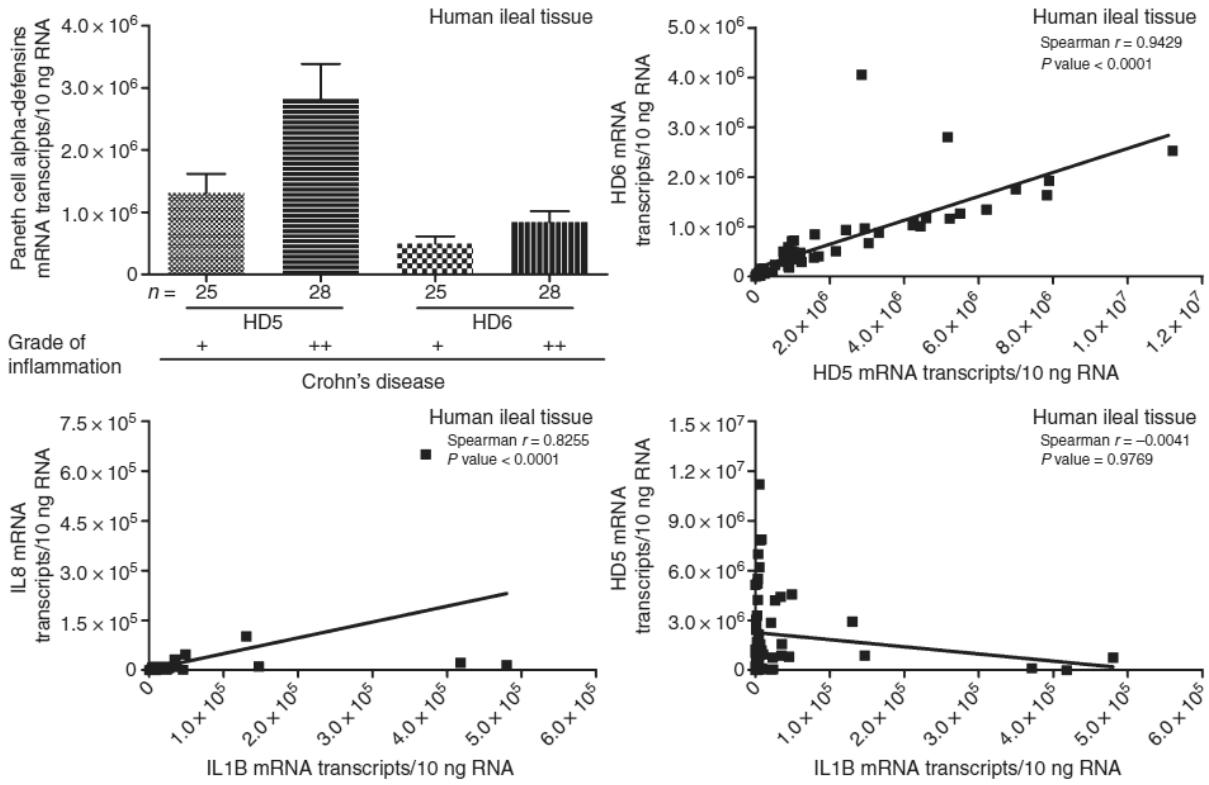

Figure 2. Inflammation severity and the expression of ileal defensins in active Crohn's disease. The mRNA levels of Paneth cell α-defensins remained unchanged comparing moderate to severely inflamed patient samples (left upper panel). The expression of both defensins strongly correlated with each other (right upper pane), but was independent of the expression of inflammatory cytokines (right lower panel). Latter exhibited matching mRNA level (left lower panel).

Table 3. Correlations of ileal mRNA expression levels

Samples ileum	IL8	IL10	Il-1b	Il-6	TNFα	ICAM1
HD5						
Spearman *r*	0.1990	0.0167	−0.004	0.0275	0.0630	0.1809
P-value	0.1532	0.9058	0.9769	0.8448	0.6540	0.1949
HD6						
Spearman *r*	0.1589	0.0236	0.0590	−0.0092	0.1093	0.1901
P-value	0.2557	0.8669	0.6764	0.9479	0.4359	0.1728
LL37						
Spearman *r*	0.4515	0.4519	0.4767	0.6544	0.5988	0.5799
P-value	0.0007	0.0007	0.0003	<0.0001	<0.0001	<0.0001
IL8						
Spearman *r*		0.4487	0.8255	0.8022	0.6204	0.7844
P-value		0.0008	<0.0001	<0.0001	<0.0001	<0.0001

Shown are the correlation scores and the respective *P*-values. We tested for correlation of HD5 and HD6 as well as LL37 mRNA level with different cytokines. We additionally performed tests to determine the strength of correlations between IL8 and different other measured cytokines.

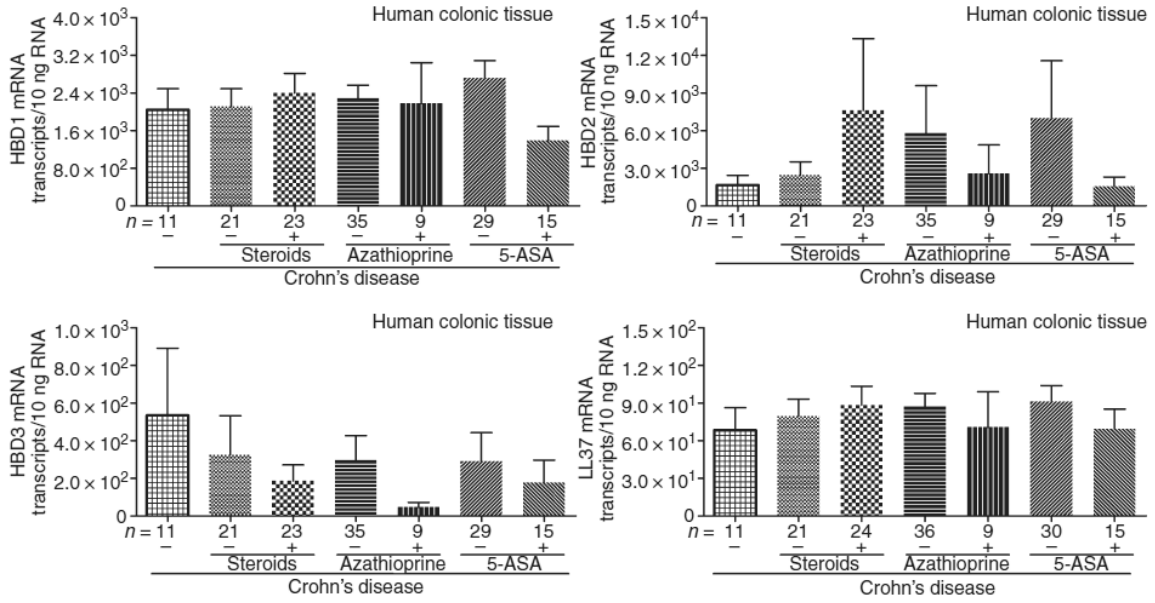

Figure 3. Current treatment and the expression of colonic defensins and LL37 in active Crohn's disease. Possible differences in the expression of inducible β-defensins as well as LL37 were not statistically significant.

mutations in the autophagy gene ATG16L1[22] and potentially by polymorphisms in the TCF-4 promotor.[23] The mRNA expression of TCF-4, a key effector of the Wnt pathway is, similarly to HD5 and HD6, specifically reduced in ileal CD. Besides directly controlling the expression of these Paneth cell α-defensins TCF-4 plays an important role in stem cell proliferation and Paneth cell maturation.[43, 44] In agreement with previous studies,[34, 41] the degree of inflammation in the mucosa did not affect HD5 and HD6 mRNA. Antithetically, in a recent Australian study, Simms *et al.*[45] suggested inflammation dependence for the decrease in ileal CD. They proposed tissue damage as the cause for the diminished levels in inflamed ileal CD mucosa. Based on their results, differences according to inflammation severity would be expected in our samples. We did not find any decrease in HD5 or HD6 in the high inflammation group compared to moderate inflammation. We also did not see a negative correlation between HD5 or HD6 mRNA levels and inflammatory cytokine expression. A reason for the unequal interpretations might be differences in study design. We only used biopsies or surgical specimens separately, whereas the Australian study mixed resection specimens and biopsies disproportionately in the different groups. In support of this idea, the Paneth cell product PLA2G2A was significantly lower in the normalized control group suggesting a possible bias because of normalization effects.[46] In summary, these data presented here further support that the decrease in Paneth cell α-defensins is seen independent of inflammation and not because of epithelial loss at the base of the crypts where Paneth cells are located.

Like in the small intestine, the different treatments did not seem to have great effects on defensin expression in the colon. HBD2 and HBD3 can be induced by proinflammatory cytokines, such as IL1B, mostly through NFκB- and AP1-dependent pathways,[47] by bacteria by means of similar pathways[31] and also by the cytokines IL-23 and IL-17.[48] NFκB has a key role in the transcriptional regulation of a proinflammatory gene programme in intestinal epithelial cells (e.g. IL-8, ICAM-1, inducible NO synthase, COX-2, and growth-related oncogene alpha). The inflammatory target genes are thereby much more affected by changes in NFκB than HBD2. The latter must be additionally regulated by factors, which have not yet been identified.[34] We observed a significant, but relatively weaker correlation between colonic HBD2 and the different cytokines, supporting the partly common but also

Table 4. Expression levels of antimicrobial peptides and cytokines: mRNA transcripts/10 ng RNA in human colonic tissue

Products	Steroids				Azathioprine				5-ASA			
Medication	–		+		–		+		–		+	
n	21		21		23		35		9		29	15
HBD1	2043 (±448.0)		2112 (±373.5)		2403 (±416.8)		2285 (±281.3)		2182 (±859.4)		2715 (±369.8)	1392 (±301.5)
HBD2	1673 (±753.3)		2470 (±1049)		7603 (±5723)		5812 (±3766)		2592 (±2269)		7006 (±4564)	1570 (±742.2)
HBD3	535.7 (±355.7)		325 (±207)		187 (±84.26)		297 (±130.3)		48.1 (±24.92)		290.7 (±151.7)	178.1 (±119.5)
LL37	68.47 (±17.65)		79.54 (±13.55)		88.39 (±15.0)		87.13 (±10.65)		70.66 (±28.43)		91.19 (±12.75)	69.31 (±15.83)
IL8	26 711 (±20 346)		10 043 (±3089)		25 238 (±11 688)		19 831 (±3861)		10 808 (±3861)		22 795 (±9351)	8688 (±3582)
IL6	754.2 (±435.0)		966.1 (±292.6)		3340 (±1393)		2436 (±929.3)		1316 (±774.7)		2046 (±940.5)	2519 (±1308)
IL10	1429 (±548.4)		1073 (±299.1)		1131 (±209.2)		1249 (±207.8)		460.2 (±127.7)		1319 (±255.5)	686.9 (±115.1)
IL1B	13432 (±5274)		14405 (±3826)		17324 (±5933)		17647 (±4368)		9258 (±3428)		17712 (±4876)	12488 (±4619)
TNFα	584.7 (±137.5)		688.0 (±111.6)		736.7 (±159.7)		731.9 (±114.7)		642 (±184.2)		714.7 (±125.3)	711.1 (±160.7)
ICAM1	3620 (±1501)		4553 (±1044)		6096 (±1625)		5634 (±1194)		4291 (±1236)		5745 (±1393)	4613 (±1048)

Shown are the means ± standard errors.

fractionally distinct regulation. Nothing is known so far about the transcriptional regulation of HBD1.

Considering the effects of corticosteroid treatment, the situation in the colon is quite complex and the conclusion based on this study design must be drawn carefully. Glucocorticoids are known to inhibit the transcription factors NFκB and AP1.[49] These are, as mentioned above, involved in the bacterial or cytokine-dependent induction of HBD2 and HBD3[31, 47], whereas HBD1 is very likely independent of them. Interestingly, in a study by Witthoft *et al.*[50] *in vitro* experiments showed an upregulation of HBD2 in Caco-2 cells following corticosteroid treatment. Similarly, in an *in vitro* study using a human epithelial lung cancer cell line, another group reported upregulation of HBD2 after dexamethason treatment.[51] These studies are compatible with the, by trend, marginal induction seen in the corticosteroid treated group and support a partly inflammatory independent regulation of HBD2.

The nucleotide analogue Azathioprine is metabolized to mercaptopurine through reduction by glutathione.[52] It is relatively lymphocyte-specific because these cells lack a salvage pathway for purine synthesis.[53] Nonetheless, as a purine-analogue blocking the synthesis of inosinic acid, it generally affects cells in the S-phase. As the intestinal epithelium undergoes cell renewal at extraordinary rates,[54, 55] an impact on the proliferating cell compartments might not be unlikely. We observed a trend for decreased expression of inducible HBDs in the azathioprine group, which did not achieve statistical significance, but potential effects can so far not completely be excluded.

It is well known that 5-ASA can modulate various inflammatory pathways (e.g. production of inflammatory cytokines, activity of inducible nitric oxide synthase, activation of NFkB).[56–58] More recently, it has been shown that 5-ASA can interfere with biological pathways that control growth and survival of colorectal cancer cells as well as colorectal mucosal cells in patients with sporadic large bowel polyps.[59, 60] If and how it has an influence on small intestinal mucosa or on healthy colonic mucosa is not clear. Mechanisms by which 5-ASA influences CRC proliferation are inhibition of both the Wnt/β-catenin pathway and epidermal growth factor receptor signalling pathways, as well as the activation of peroxisome proliferator-activated receptor-γ.[61] We see no difference in ileal defensin expression following 5-ASA treatment and no significant changes in the colonic expression of

Table 5. Correlations of colonic mRNA expression levels

Samples colon	IL8	IL10	IL1B	IL6	TNFα	ICAM1
HBD1						
Spearman *r*	0.1632	0.1209	0.1563	0.1151	0.2135	0.2307
P-value	0.2898	0.4456	0.3109	0.4569	0.1641	0.1320
HBD2						
Spearman *r*	0.5185	0.2965	0.5838	0.4305	0.4418	0.5386
P-value	0.0003	0.0536	<0.0001	0.004	0.0034	0.0002
IL8						
Spearman *r*		0.3022	0.8469	0.9005	0.6881	*0.8513*
P-value		0.0436	<0.0001	<0.0001	<0.0001	<0.0001

Shown are the correlation scores and the respective *P*-values. We tested for correlation of HBD1 and HBD2 as well as LL37 mRNA level with different cytokines. We additionally performed tests to determine the strength of correlations between IL8 and different other measured cytokines.

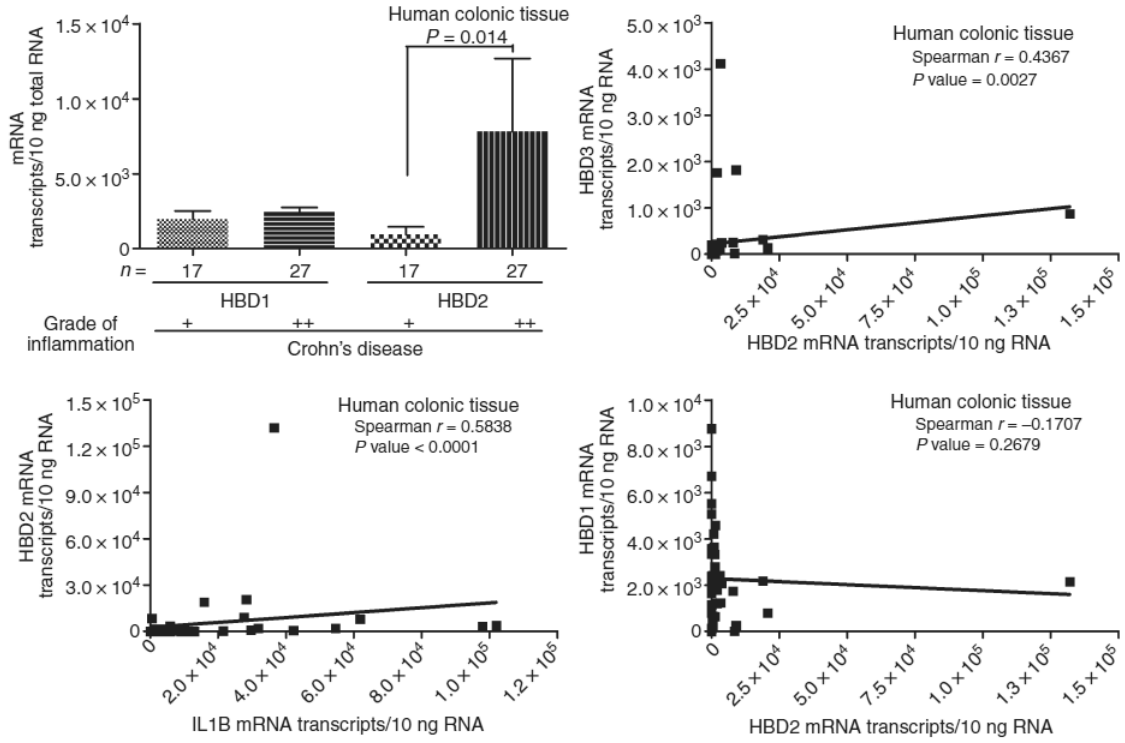

Figure 4. Inflammation severity and the expression of colonic defensins in active Crohn's disease. The constitutively expressed beta defensin HBD1 exhibited unchanged mRNA level after stratifying according to inflammation severity. Differently, the inducible HBD2 was significantly higher expressed in the severely inflamed tissue samples (left upper panel) correlating with inflammatory cytokines, e.g. IL1B (lower panel left) and HBD3 (upper panel right).

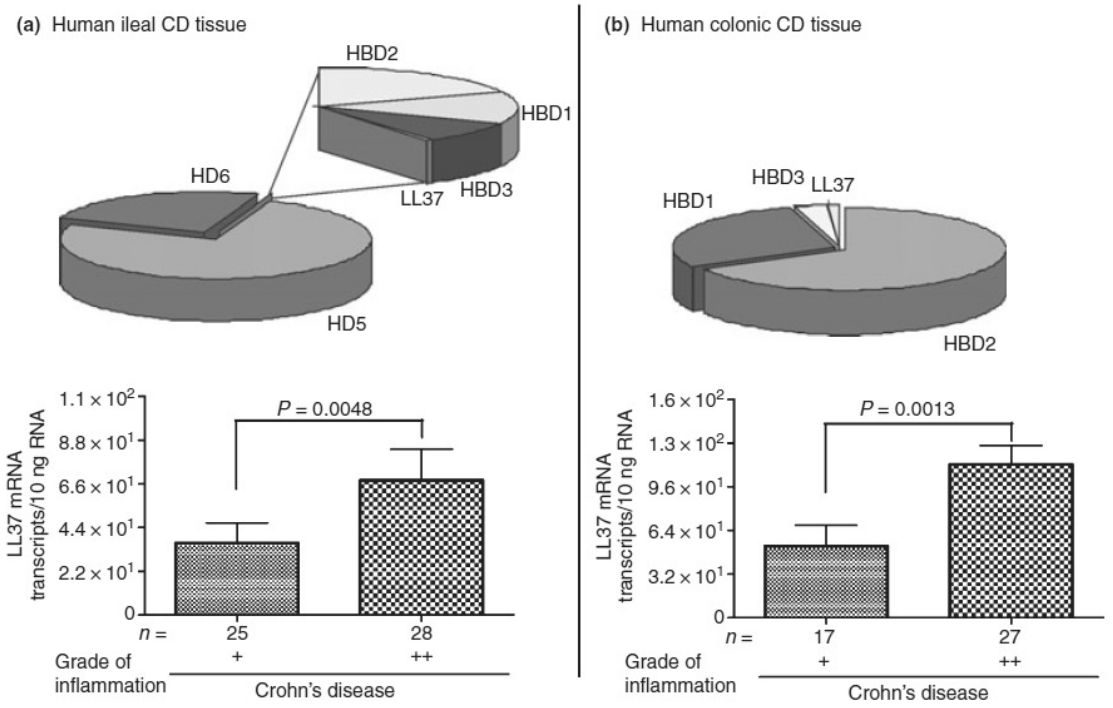

Figure 5. Distribution of ileal and colonic antimicrobial peptides in active Crohn's disease. (a) upper panel: comparison of mRNA levels of defensins and LL37 in ileal active Crohn's disease; lower panel: Inflammation severity and the expression of ileal LL-37 in active Crohn's disease. The Paneth cell α-defensins HD5 and HD6 are clearly the dominating members of the measured antimicrobial factors. HBD2 seems to be more prominently expressed than HBD1 in the studied active CD samples. According to mRNA levels, LL37 was significantly highly expressed in the severely inflamed tissue samples (lower panel). (b) upper panel: comparison of mRNA levels of defensins and LL37 in colonic active Crohn's disease; lower panel: Inflammation severity and the expression of colonic LL-37 in active Crohn's disease. Comparable to the results in the ileum, HBD2 seems to have a more prominent role in the studied active CD samples than HBD1. Also matching to the ileal situation, mRNA levels of LL37 were significantly higher in the severely inflamed tissue samples (lower panel).

inducible β-defensins. However, we could observe lower mRNA levels of HBD1 in the colon, comparing 5-ASA treated patients with those who did not receive this medication. The effect did not hold up after *P*-value adjustment.

Stratifying the patients according to IL8, we found that an increase in colonic HBD2 expression in severe inflammation conforms to the significant induction in the ileum in the respective group. The same was seen for LL37. This antimicrobial peptide is known to have an influence on innate immune responses, angiogenesis and wound healing[62] and dysfunctions of LL37 emerge as a central factor in the pathogenesis of several cutaneous diseases.[63] There are different reports available on the influence of inflammation and inflammatory cytokines on LL37 expression, with some rebutting a role,[64] but others supporting the idea.[65, 66] Supporting an inflammatory controlled regulation, we found a correlation of LL-37 with inflammatory cytokines and an increase with more inflammation.

In conclusion, currently used standard treatments including corticosteroids, azathioprine and 5-ASA did not significantly influence the overall α-defensin expression in the terminal ileum as well as colonic β-defensin expression and LL37 mRNA level. A possible limitation of the study is that most patients received multiple treatments and thus the results are based on a cross-sectional design as well as lacking information on protein level. Further limitations might be that the study was conducted in a clinical patient – rather than in an experimental animal setting, which

limits the possibility of drawing ultimate conclusions. The patients were often transferred to our tertiary care centre by practising physicians and therefore sometimes received therapies like 5-ASA, which are not necessarily evidence-based[5]

For future treatment of IBD, strategies might aim to strengthen protective innate immunity. In this context, the reported efficiency of *Trichuris suis* therapy in CD provokes the testable question of whether the stimulation of defensins by parasitic worms might be an explanation for their therapeutic effect. Probiotic bacteria like *Escherichia coli* bacteria and *Lactobacilli*, have been shown to induce antimicrobial peptides *in vitro* and *in vivo*.[30, 32, 34] This might be an important mechanism to support the mucosa in preventing bacterial invasion. Probiotic bacteria are the first therapeutic agents for IBD of which we know that they bolster the production of antimicrobial peptides, but it is likely that others could have similar effects. In the future, further therapeutic strategies aimed at restoring the host–microbe balance at the intestinal mucosa should be identified and may prove superior to those that broadly suppress inflammation and adaptive immunity.

ACKNOWLEDGEMENTS

We thank the members of the Robert-Bosch-Hospital for recruitment of patients and for the specimens. We thank K. Siegel and J. Bader for excellent technical assistance. *Declaration of personal interests*: None. *Declaration of funding interests*: The study was supported by the Robert Bosch Foundation (Stuttgart, Germany), the Deutsche Forschungsgemeinschaft (DFG) and the DCCV (German patient organization of Crohn's disease and ulcerative colitis). Jan Wehkamp is an Emmy Noether scholar of the Deutsche Forschungsgemeinschaft (WE 436/1-1).

REFERENCES

1 Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417–29.
2 Halfvarson J, Jess T, Magnuson A, *et al.* Environmental factors in inflammatory bowel disease: a co-twin control study of a Swedish-Danish twin population. *Inflamm Bowel Dis* 2006; **12**: 925–33.
3 Strober W. Immunology. Unraveling gut inflammation. *Science* 2006; **313**: 1052–4.
4 Levine AD, Fiocchi C. Immunology of inflammatory bowel disease. *Curr Opin Gastroenterol* 2000; **16**: 306–9.
5 Travis SP, Stange EF, Lemann M, *et al.* European evidence based consensus on the diagnosis and management of Crohn's disease: current management. *Gut* 2006; **55**(Suppl 1): i16–35.
6 Lemann M, Mary JY, Duclos B, *et al.* Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054–61.
7 Duchmann R, May E, Heike M, Knolle P, Neurath M, Meyer zum Büschenfelde K-H. T cell specifity and cross reactivity towards enterobacteria, *Bacteroides*, *Bifidobacterium*, and antigens from resident intestinal flora in humans. *Gut* 1999; **44**: 812–8.
8 Sartor RB. Intestinal microflora in human and experimental inflammatory bowel disease. *Curr Opin Gastroenterol* 2001; **17**: 324–30.
9 MacPherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* 1996; **38**: 365–75.
10 Fellermann K, Wehkamp J, Herrlinger KR, Stange EF. Crohn's disease: a defensin deficiency syndrome? *Eur J Gastroenterol Hepatol* 2003; Jun; **15**(6): 627–34.
11 Meyer-Hoffert U, Hornef MW, Henriques-Normark B, *et al.* Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut* 2008; **57**: 764–71.
12 Bevins CL, Nita H, Salzman NH, Ghosh D, Huttner KM. Human defensin-5 (HD-5) transgenic mice:paneth cell expression and protection from lethal salmonella typhimurium infection. *Gastroenterology* ????; 122(Suppl. 1): 2 A.D.
13 Swidsinski A, Ladhoff A, Pernthaler A, *et al.* Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 44–54.
14 Rhodes JM. The role of *Escherichia coli* in inflammatory bowel disease. *Gut* 2007; **56**: 610–2.
15 Darfeuille-Michaud A, Boudeau J, Bulois P, *et al.* High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004; **127**: 412–21.
16 Wehkamp J, Koslowski M, Wang G, Stange EF. Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease. *Mucosal Immunol* 2008; 1(Suppl. 1): S67–74.
17 Wehkamp J, Salzman NH, Porter E, *et al.* Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A* 2005; **102**: 18129–34.
18 Wehkamp J, Chu H, Shen B, *et al.* Paneth cell antimicrobial peptides: topographical distribution and quantification in human gastrointestinal tissues. *FEBS Lett* 2006; **580**: 5344–50.
19 George MD, Wehkamp J, Kays RJ, *et al.* In vivo gene expression profiling of human intestinal epithelial cells: analysis by laser microdissection of formalin fixed tissues. *BMC Genomics* 2008; **9**: 209. **209.**
20 Ogura Y, Bonen DK, Inohara N, *et al.* A frameshift mutation in NOD2 associated with susceptibility to Crohn's diease. *Nature* 2001; **411**: 603–6.
21 Wehkamp J, Harder J, Weichenthal M, *et al.* NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. *Gut* 2004; **53**: 1658–64.

22 Cadwell K, Liu JY, Brown SL, *et al.* A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* 2008; **456**: 259–63.

23 Koslowski MJ, Kubler I, Chamaillard M, *et al.* Genetic variants of Wnt transcription factor TCF-4 (TCF7L2) putative promoter region are associated with small intestinal Crohn's disease. *PLoS ONE* 2009; **4**: e4496.

24 Kapel N, Benahmed N, Morali A, *et al.* Fecal beta-defensin-2 in children with inflammatory bowel diseases. *J Pediatr Gastroenterol Nutr* 2009; **48**: 117–20.

25 Wehkamp J, Harder J, Weichenthal M, *et al.* Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis* 2003; **9**: 215–23.

26 Nuding S, Fellermann K, Wehkamp J, Stange EF. Reduced mucosal antimicrobial activity in Crohn's disease of the colon. *Gut* 2007; **56**: 1240–7.

27 Fellermann K, Stange DE, Schaeffeler E, *et al.* A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am J Hum Genet* 2006; **79**: 439–48.

28 Ogushi KF, Wada AF, Niidome TF, *et al.* Salmonella enteritidis FliC (flagella filament protein) induces hu beta-defensin-2 mRNA production by Caco-2 cells. *J Biol Chem* 2001; **276**: 30521–6.

29 Wehkamp J, Schmidt K, Herrlinger KR, *et al.* Defensin pattern in chronic gastritis: HBD-2 is differentially expressed with respect to Helicobacter pylori status. *J Clin Pathol* 2003; **56**: 352–7.

30 Mondel M, Schroeder BO, Zimmermann K, *et al.* Probiotic E. coli treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. *Mucosal Immunol* 2008; **2**: 166–72.

31 Schlee M, Wehkamp J, Altenhoefer A, Oelschlaeger TA, Stange EF, Fellermann K. The induction of human beta-defensin-2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. *Infect Immun* 2007; **75**(5): 2399–407.

32 Schlee M, Harder J, Koten B, Stange EF, Wehkamp J, Fellermann K. Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. *Clin Exp Immunol* 2008; **151**: 528–35.

33 Schultz M, Lindstrom AL. Rationale for probiotic treatment strategies in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* 2008; **2**: 337–55.

34 Wehkamp J, Harder J, Wehkamp K, *et al.* NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun* 2004; **72**: 5750–8.

35 Hosaka Y, Koslowski M, Nuding S, *et al.* Antimicrobial host defense in the upper gastrointestinal tract. *Eur J Gastroenterol Hepatol* 2008; **20**: 1151–8.

36 Holzer A, Harsch S, Renner O, *et al.* Diminished expression of apical sodium-dependent bile acid transporter in gallstone disease is independent of ileal inflammation. *Digestion* 2008; **78**: 52–9.

37 Gasche C, Grundtner P. Genotypes and phenotypes in Crohn's disease: do they help in clinical management? *Gut* 2005; **54**: 162–7.

38 Wehkamp J, Salzman NH, Porter E, *et al.* Reduced Paneth cell {alpha}-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A* 2005; **102**: 18129–34.

39 Kobayashi KS, Chamaillard M, Ogura Y, *et al.* Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005; **307**: 731–4.

40 Elphick D, Liddell S, Mahida YR. Impaired luminal processing of human defensin-5 in crohn's disease: persistence in a complex with chymotrypsinogen and trypsin. *Am J Pathol* 2008; **172**: 702–13.

41 Wehkamp J, Fellermann K, Herrlinger KR, Bevins CL, Stange EF. Mechanisms of disease: defensins in gastrointestinal diseases. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 406–15.

42 Fishbein T, Novitskiy G, Mishra L, *et al.* NOD2-expressing bone marrow-derived cells appear to regulate epithelial innate immunity of the transplanted human small intestine. *Gut* 2008; **57**: 323–30.

43 Wehkamp J, Wang G, Kubler I, *et al.* The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J Immunol* 2007; **179**: 3109–18.

44 van EsJH, Jay P, Gregorieff A, *et al.* Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat Cell Biol* 2005; **7**: 381–6.

45 Simms LA, Doecke JD, Walsh MD, Huang N, Fowler EV, Radford-Smith GL. Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut* 2008; **57**: 903–10.

46 Bevins CL, Stange EF, Wehkamp J. Decreased Paneth cell defensin expression in ileal Crohn's disease is independent of inflammation, but linked to the NOD2 1007fs genotype. *Gut* 2009; **58**: 882–3.

47 O'Neil DA, Porter EM, Elewaut D, *et al.* Expression and regulation of the human β-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol* 1999; **163**: 6718–24.

48 Wilson NJ, Boniface K, Chan JR, *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007; **8**: 950–7.

49 Kovalovsky D, Refojo D, Holsboer F, Arzt E. Molecular mechanisms and Th1/Th2 pathways in corticosteroid regulation of cytokine production. *J Neuroimmunol* 2000; **109**: 23–9.

50 Witthoft T, Pilz CS, Fellermann K, Nitschke M, Stange EF, Ludwig D. Enhanced human beta-defensin-2 (hBD-2) expression by corticosteroids is independent of NF-kappaB in colonic epithelial cells (CaCo2). *Dig Dis Sci* 2005; **50**: 1252–9.

51 Homma T, Kato A, Hashimoto N, *et al.* Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. *Am J Respir Cell Mol Biol* 2004; **31**: 463–9.

52 Teml A, Schaeffeler E, Herrlinger KR, Klotz U, Schwab M. Thiopurine treatment in inflammatory bowel disease: clinical pharmacology and implication of pharmacogenetically guided dosing. *Clin Pharmacokinet* 2007; **46**: 187–208.

53 Atreya I, Neurath MF. Azathioprine in inflammatory bowel disease: improved molecular insights and resulting clinical implications. *Expert Rev Gastroenterol Hepatol* 2008; **2**: 23–34.

54 Crosnier C, Stamataki D, Lewis J. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* 2006; **7**: 349–59.

55 Gregorieff A, Clevers H. Wnt signaling in the intestinal epithelium: from endoderm to cancer. *Genes Dev* 2005; **19**: 877–90.

56 Bantel H, Berg C, Vieth M, Stolte M, Kruis W, Schulze-Osthoff K. Mesalazine inhibits activation of transcription factor NF-kappaB in inflamed mucosa of patients with ulcerative colitis. *Am J Gastroenterol* 2000; **95**: 3452–7.

57 Kaiser GC, Yan F, Polk DB. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes. *Gastroenterology* 1999; **116**: 602–9.

58 Mahida YR, Lamming CE, Gallagher A, Hawthorne AB, Hawkey CJ. 5-Aminosalicylic acid is a potent inhibitor of interleukin 1 beta production in organ culture of

colonic biopsy specimens from patients with inflammatory bowel disease. *Gut* 1991; **32**: 50–4.

59 Reinacher-Schick A, Seidensticker F, Petrasch S, *et al.* Mesalazine changes apoptosis and proliferation in normal mucosa of patients with sporadic polyps of the large bowel. *Endoscopy* 2000; **32**: 245–54.

60 Reinacher-Schick A, Schoeneck A, Graeven U, Schwarte-Waldhoff I, Schmiegel W. Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis* 2003; **24**: 443–51.

61 Stolfi C, Pellegrini R, Franze E, Pallone F, Monteleone G. Molecular basis of the potential of mesalazine to prevent colorectal cancer. *World J Gastroenterol* 2008; **14**: 4434–9.

62 Otte JM, Zdebik AE, Brand S, *et al.* Effects of the cathelicidin LL-37 on intestinal epithelial barrier integrity. *Regul Pept* 2009; **156**: 1–3.

63 Schauber J, Gallo RL. Antimicrobial peptides and the skin immune defense system. *J Allergy Clin Immunol* 2008; **122**: 261–6.

64 Hase K, Eckmann L, Leopard JD, Varki N, Kagnoff MF. Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. *Infect Immun* 2002; **70**: 953–63.

65 Peric M, Koglin S, Kim SM, *et al.* IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J Immunol* 2008; **181**: 8504–12.

66 Schauber J, Rieger D, Weiler F, *et al.* Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases. *Eur J Gastroenterol Hepatol* 2006; **18**: 615–21.

3.4 General Discussion and Outlook

3.4.1 Genetic analysis in IBD

The current understanding of IBD pathogenesis is that of a complex interplay of genetic and environmental factors leading to an impaired mucosal barrier and subsequent inflammatory immune responses towards intestinal bacteria. Data from epidemiological, molecular, and genome-wide association studies (GWAS) suggest that UC and CD are, to a certain extent, related polygenic diseases but also differ in specific genetic associations. Even though the earlier autoimmune disease concept for CD has been revised in the last years, the disorder is still often genetically analyzed in combination with diseases like multiple sclerosis or rheumatoid arthritis. A study by Sirota et al., investigated the homogeneity of genetic architectures across different autoimmune diseases within multiple GWAS (Sirota et al., 2009). The researchers compared genetic variation profiles of six autoimmune diseases, in which they included CD, as well as five diseases which they classified as non-autoimmune. Much in support of the more recent barrier defect pathogenesis concept, they found that CD was similar to none of the other five investigated autoimmune disorders and therefore not further discussed within this group. In general they also concluded that detecting differences between diseases which were previously thought to be similar may point out new and specific candidate genes and pathways. We think this is also valid for disease subgroups which show specific phenotypes within a common trait, and especially in a symptomatically heterogenic disorder such as CD. The identification of subgroup specific candidate genes will improve our understanding of the underlying pathogenesis and help to explain why some patients have only a certain location affected, an especially early symptom development or a highly aggravated disease course.

In the last years, the utilization of GWAS, which test for association between hundreds of thousands of SNPs and a given phenotype, allowed the successful identification of various IBD risk variants. 99 such susceptibility loci are to date identified and/or

validated by GWAS: 71 associated with Crohn's disease, 47 with ulcerative colitis, and 28 with both inflammatory bowel diseases (Lees et al., 2011). In the GWAS context, significant SNP associations have to exceed a conservative threshold (usually $P < 5 \times 10–8$) and survive a replication in independent cohorts. A general problem of this hypothesis free approach though, is the lack of identified causative variants. Usually genome wide studies can narrow in susceptibility regions or genes but to truly understand the genetics that underlie a disease trait, it requires further fine mapping to highlight the causative variant within a risk region and often additional functional studies on identified candidate genes. This can be nicely demonstrated by means of the first identified CD associated gene *NOD2*. As early as 1996 a genome-wide search identified a putative CD-susceptibility locus on chromosome 16 (Hugot et al., 1996). It took until 2001 to identify a frameshift variant and two missense variants in *NOD2* as three independent associations for CD within this locus (Hugot et al., 2001). Since then, various studies were able to clear up the functional aspects of the *NOD2* mutations in CD susceptibility, but investigations are still ongoing. In addition to the often missing causal associations, another general controversy in GWAS approaches criticizes the fact that discovered variants generally only capture a few percent of an estimated heritability for investigated complex traits (Witte, 2010; Gibson, 2011). A possible explanation for this discrepancy might be the contributions of still unknown rare variants, epistasis, epigenetics and genotype–environment interactions, which are yet to be identified. On the other hand, it might imply that complex traits including IBD are truly affected by thousands of small effect variants and/or that GWAS might simply miss some, if not many, associations. Since it is unlikely that GWAS will ever have sufficient statistical power to reveal the complete picture of complex trait heritability and due to their stringent character, it is clear that the lack of evidence for an association or replication in a GWAS, does not generally postulate its absence (McCarthy et al., 2008; Gibson, 2011). Interestingly a recent study by Amos et al. investigated the efficiency of GWAS versus target gene approaches in case-control studies for infectious diseases. They found that the candidate gene approach's greater statistical power might identify causal variants which otherwise would be missed

(Amos et al., 2011). Similar to the therein investigated populations, a control group for IBD might contain individuals with susceptible genotypes who have yet not been exposed to known or so far unknown environmental risks. Such a bias due to complex gene-environment interplay in the disease might play a role in IBD genetic research. It deserves more thought regarding IBD heredity, even though such a bias will be hard to eliminate. Another challenge for future genetic studies of complex diseases will be the further reintroduction of biology (McCarthy et al., 2008). An efficient identification of susceptibility loci might benefit from an integration of statistical evidence with some assessment of functional importance. Embracing aspects of biological candidacy e.g., variants mapping to genes that play a role within the same pathways as previously implicated susceptibility genes, could help to identify additional, so far disregarded risk factors.

In conclusion, GWAS have not been able to fully explain the genetic predisposition for IBD as well as for other excessively studied multifactorial and genetically complex diseases. We take that as evidence that additional, non genome wide studies are needed, which, like the herein presented work, focus on disease involved pathways and already identified pathogenesis processes.

3.4.2 Genetic associations linking Paneth cells to ileal CD

The major role of the specialized Paneth cell in ileal CD has been badged by different functional defects and disease associated genes which are linked to the cells antimicrobial function. In addition to the already mentioned intracellular MDP receptor *NOD2* (Ogura et al., 2001; Wehkamp et al., 2004b), other PC relevant genes, "*ATG16 autophagy related 16-like 1" (ATG16L1)* (Cadwell et al., 2008), *X-box binding protein 1 (XBP1)* (Kaser et al., 2008), and just recently the "*intermediate conductance calcium-activated potassium channel protein"* (*KCNN4*) (Simms et al., 2010) have been implicated in the disease. Defects in *NOD2* could be liaised with PC α- defensin deficiency in humans and mice and represent the foremost genetic ileal CD susceptibility factor with a link to Paneth cell innate immunity (Wehkamp et al., 2005b;

Wehkamp et al., 2008; Kobayashi et al., 2005). Like *NOD2*, *ATG16L1* shows an increased effect in the ileal CD subphenotype (Hampe et al., 2007; Fowler et al., 2008) and is likely relevant in a similar context. The *ATG16L1* risk variant is accompanied by PC abnormalities in patients, which are correspondingly present in ATG16L1^{HM} mice (Cadwell et al., 2008). The gene encodes a bona fide autophagy protein which is also essential in granule exocytosis pathways, respectively peptide export in secretory cells. The maintenance of secretory cells like the AMP producing Paneth Cell is furthermore influenced by the transcription factor XBP1, a key component of the endoplasmic reticulum (ER) stress response (Acosta-Alvear et al., 2007). All secretory cells rely on the capacity of the ER to fold, modify and traffic polypeptides. XBP1 activates ER biogenesis and the transcription of chaperone genes. Its deletion in mouse intestinal epithelial cells results in spontaneous enteritis and increased colitis susceptibility secondary to PC dysfunction and increased epithelial reactivity to bacterial products and TNFα (Kaser et al., 2008). Another CD associated factor, the *KCNN4* encoded K_{Ca}3.1 protein has an important function in T lymphocyte Ca(2+) signaling and, like ATG16L1, a role in Paneth cell secretion. Besides its genetic association with the disease, it also exhibits significantly reduced mRNA levels in *NOD2* mutated patients, highlighting a rather complex genetic interplay in CD pathogenesis. A previous study could show that the loss of K_{Ca}3.1 protects mice from severe colitis in two IBD models, so the therapeutic use of pharmacologic K_{Ca}3.1 inhibitors has been suggested (Di et al., 2010). The data from the more recent human study contradicts a potential use as it might hint to an impaired rather than increased function. Investigations on the effect of *KCNN4* defects on antimicrobial PC function could provide promising approaches to a better understanding of K_{Ca}3.1 mediated disease mechanisms. Such studies as well as projects focusing on the interplay of the different genetic hits: *ATG16L1, NOD2, XBP1, KCNN4* and now *TCF7L2* and *LRP6* are especially needed to identify potential therapeutic targets and to clear up symptomatic heterogeneity in patients. The now genetically confirmed role of the PC maturation factor *TCF7L2* in ileal CD hints to impaired cell development as a new disease concept with a likely major role of the Wnt pathway. The association of a coding *LRP6* variant with an early onset and also

penetrating ileal CD subphenotype and the decreased mRNA expression of this important canonical Wnt co-receptor complement the picture. Two genes, *DLG5* and *E- cadherin* (*CDH1*) which have also been introduced in the context of CD susceptibility provide an additional bridge to disturbed canonical Wnt activity (Muise et al., 2009; Nechiporuk et al., 2007; Stoll et al., 2004). Even though those genes have not directly been linked to Paneth cell function, their roles in β- catenin localization would support an influence on canonical signaling activity and a hypothetical subsequent impact on the expression of HD5 and -6.

Taken together, factors in pathways involved in the recognition of bacteria, stress responses or secretory processes in Paneth cells show impairments in ileal CD. Those defects likely act together and result in a compromised antimicrobial function in affected patients. The transcription factor *TCF7L2* and the Wnt co-receptor *LRP6* may now be added as novel candidates affecting the Paneth cell in ileal CD. These and the multiple other associations linked to ileal CD and the Paneth cell function support a primary rather than secondary role of impaired antimicrobial defenses (Bevins et al., 2009). Most importantly, the diminished expression of the innate HD5 and -6 antimicrobial peptides provide, as already mentioned, an attractive and direct alternative target (Wang et al., 2007) to the current anti-inflammatory approaches in CD therapy.

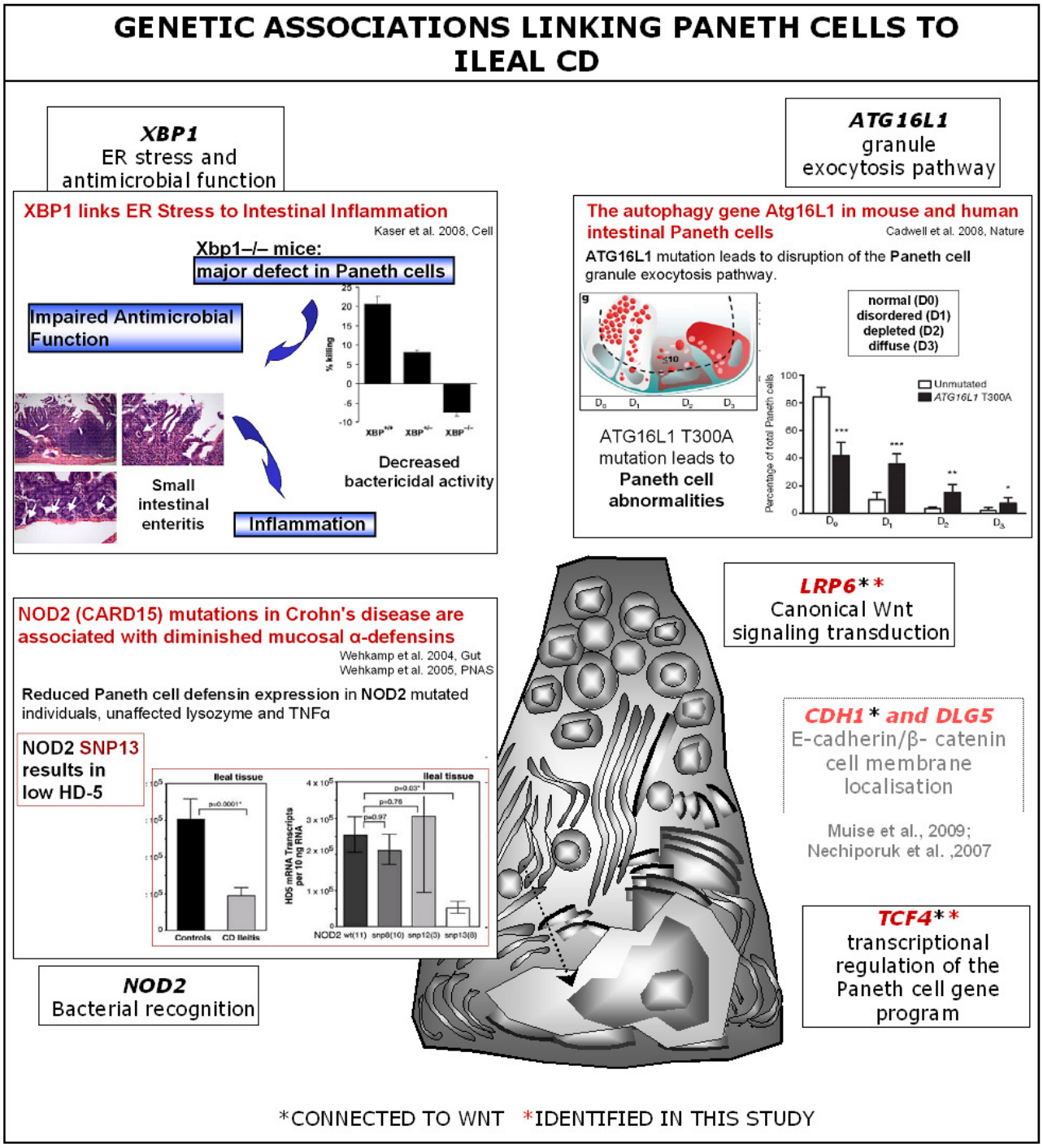

Figure 3.6: **Overview on multiple disease susceptibility genes with an involvement in Paneth cell biology**: The NOD2 3020insC frameshift mutation could be associated with especially reduced HD5 and -6 mRNA levels in patients. Morphological abnormalities in Paneth cells are present in patients homozygous for the ATG16L1 T300A disease risk allele and decreased antimicrobial function has been reported in mice depleted of XBP1, a transcription factor which could also be genetically liaised with the disease in humans. Introduction of the rs3814570 risk variant of the TCF7L2 putative promotor and the early onset associated LRP6 Ile1062Val mutation support a disease underlying mechanism of diminished canonical Wnt activity in ileal CD and an involvement of impaired cell differentiation. E- cadherin and

DLG5, which both influence canonical Wnt via the regulation of β- catenin localization, were genetically associated with CD, but a direct effect on Paneth cells has not yet been investigated. Interestingly, the *KCNN4* coded potassium channel $K_{Ca}3.1$ has just recently been introduced in this context of CD susceptibility and again provides a link to Paneth cell function as it regulates secretory processes in this cell.

3.4.3 Outlook

Despite modern treatment, there is still no terminal cure for Crohn's Disease. This is likely in part due to a lack of adequate targets for medical manipulation and highlights the need for a better understanding of disease causes and pathogenesis mechanisms. Via the evaluation of genetic associations of canonical Wnt pathway key factors in the IBD subgroup of small intestinal Crohn's Disease, it was now possible to identify new candidates within this context. Affirming the involvement of disturbed Wnt signaling as well as the other herein collected data on HD5 and -6 highly supports previous investigations on the critical role of the Paneth cell in small intestinal CD. Additionally, the discussed results on two canonical Wnt pathway factors hint to a potential disturbance of cell differentiation and/or proliferation as a new and likely relevant disease related process. In addition to genetic investigations on further Wnt pathway factors in the context of small intestinal CD susceptibility, future experimental studies could include:

1.) A concerted action to functionally characterize the confirmed candidate genes in connection with the disease. This could be done in adequate animal model systems or by employing *in vitro* approaches ideally in the context of inflammation and or bacterial invasion with regards to antimicrobial defenses and the regenerative potential of intestinal epithelial cells.

2.) Another future aim should be an integration of so far known disease mechanisms with the reported new insights followed by investigations on epistatic effects and gene-environment interplay. In this regard it seems indispensable to implement

adequate software tools and biostatistical platform technologies, as otherwise important interactions or regulatory circuits might be missed.

3.) Disease associated SNPs should additionally be evaluated regarding a potential diagnostic value. They should therefore be subjected to a conjunction with environmental risk factors and clinical indices and investigated in a prospective fashion. An adequately high amount of patients would need to be phenotyped at clinical sites, genotyped for the most robust risk variants and monitored over a minimum of 3 years. A standardized clinical data set would have to use state-of-the-art variables. Clinical and genetic risk indicators and the algorithmic combinations of this novel data set should evaluate a predictive power regarding therapy response, maintenance of remission as well as disease progression and/or outcome. Due to a limitation in study time, the evaluation of the last two parameters would only partly achieve explanatory power. Nonetheless, the gained data might provide valuable information on the role of genetic indicators for the diagnosis and management of risk patients.

4.) The identification and evaluation of putative manipulative compounds, which bolster LRP6 function and/or intestinal Wnt pathway activity in general, would provide a first step towards the translation of scientific results into the development of causal treatment for affected patients. As mentioned in the discussion, R-Spondin1 might be a testable candidate. Its consideration can nonetheless only follow more detailed investigations on the mechanistic and functional conditions in patients as well as tests in animal models and *in vitro* systems.

The herein described results represent a first step towards new insights into disease pathogenesis and progression of small intestinal CD. We hope they will pave the way for future and additional studies on the role of IBD subtype specific heredity, the influence of Wnt signaling in ileal CD and the importance of antimicrobial defense mechanisms at the intestinal barrier. Finally, we hope that the presented data and

subsequent investigations will ultimately lead to new impulses for the development of targeted diagnostics and therapy.

4. REFERENCE LIST

Aberle,H., Bauer,A., Stappert,J., Kispert,A., and Kemler,R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. *16*, 3797-3804.

Acosta-Alvear,D., Zhou,Y., Blais,A., Tsikitis,M., Lents,N.H., Arias,C., Lennon,C.J., Kluger,Y., and Dynlacht,B.D. (2007). XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. Mol. Cell *27*, 53-66.

Akira,S., Uematsu,S., and Takeuchi,O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783-801.

Aldhous, M.C., Abu Bakar, S., Prescott, N.J., Palla, R., Soo, K., Mansfield, J.C., Mathew, C.G., Satsangi, J., Armour, J.A.. Measurement methods and accuracy in copy number variation: failure to replicate associations of beta-defensin copy number with Crohn's disease.

Amann,J.M., Chyla,B.J., Ellis,T.C., Martinez,A., Moore,A.C., Franklin,J.L., McGhee,L., Meyers,S., Ohm,J.E., Luce,K.S., Ouelette,A.J., Washington,M.K., Thompson,M.A., King,D., Gautam,S., Coffey,R.J., Whitehead,R.H., and Hiebert,S.W. (2005). Mtgr1 is a transcriptional corepressor that is required for maintenance of the secretory cell lineage in the small intestine. Mol. Cell Biol. *25*, 9576-9585.

Amos W., Driscoll E., Hoffman J.I. (2011). Candidate genes versus genome-wide associations: which are better for detecting genetic susceptibility to infectious disease? Proc Biol Sci. *278*, 1183-8.

Andreu,P., Colnot,S., Godard,C., Gad,S., Chafey,P., Niwa-Kawakita,M., Laurent-Puig,P., Kahn,A., Robine,S., Perret,C., and Romagnolo,B. (2005). Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development *132*, 1443-1451.

Andreu,P., Peignon,G., Slomianny,C., Taketo,M.M., Colnot,S., Robine,S., Lamarque,D., Laurent-Puig,P., Perret,C., and Romagnolo,B. (2008). A genetic study of the role of the Wnt/beta-catenin signaling in Paneth cell differentiation. Dev. Biol. *324*, 288-296.

Arce,L., Yokoyama,N.N., and Waterman,M.L. (2006). Diversity of LEF/TCF action in development and disease. Oncogene *25*, 7492-7504.

Avery, MacLeod, and McCarty (1944). Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types: Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type III. Journal of Experimental Medicine ***79*** , 137–58.
Ayabe,T., Satchell,D.P., Wilson,C.L., Parks,W.C., Selsted,M.E., and Ouellette,A.J. (2000). Secretion of microbicidal alpha-defensins by intestinal Paneth cell response to bacteria. Nat. Immunol. *1*, 113-118.

Bafico,A., Liu,G., Yaniv,A., Gazit,A., and Aaronson,S.A. (2001). Novel mechanism of Wnt signaling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat. Cell Biol. *3*, 683-686.

Barker,N., van de,W.M., and Clevers,H. (2008). The intestinal stem cell. Genes Dev. *22*, 1856-1864.

Barker,N., van Es,J.H., Kuipers,J., Kujala,P., van den,B.M., Cozijnsen,M., Haegebarth,A., Korving,J., Begthel,H., Peters,P.J., and Clevers,H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003-1007.

Barton,N.H. 2011. Estimating linkage disequilibria. Heredity *106*,205-6.

Begue,B., Dumant,C., Bambou,J.C., Beaulieu,J.F., Chamaillard,M., Hugot,J.P., Goulet,O., Schmitz,J., Philpott,D.J., Cerf-Bensussan,N., and Ruemmele,F.M. (2006). Microbial induction of CARD15 expression in intestinal epithelial cells via toll-like receptor 5 triggers an antibacterial response loop. J Cell Physiol *209*, 241-252.

Bentley,R.W., Pearson,J., Gearry,R.B., Barclay,M.L., McKinney,C., Merriman,T.R., and Roberts,R.L. (2009). Association of Higher DEFB4 Genomic Copy Number With Crohn's Disease. Am. J Gastroenterol. *105*, 354-9.

Bernstein,C.N. (2006). Inflammatory bowel diseases as secondary causes of osteoporosis. Curr. Osteoporos. Rep. *4*, 116-123.

Bevins,C., Porter,E.M., and Ganz,T. (1999). Defensins and innate host defence of the gastrointestinal tract. Gut *45*, 911-915.

Bevins,C.L. (2004). The Paneth cell and the innate immune response. Curr Opin Gastroenterol *20*, 572-580.

Bevins,C.L., Salzman,N.H. (2011) Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol. *9*, 356-68.

Bevins,C.L., Stange,E.F., and Wehkamp,J. (2009). Decreased Paneth cell defensin expression in ileal Crohn's disease is independent of inflammation, but linked to the NOD2 1007fs genotype. Gut *58*, 882-883.

Binnerts,M.E., Kim,K.A., Bright,J.M., Patel,S.M., Tran,K., Zhou,M., Leung,J.M., Liu,Y., Lomas,W.E., III, Dixon,M., Hazell,S.A., Wagle,M., Nie,W.S., Tomasevic,N., Williams,J., Zhan,X., Levy,M.D., Funk,W.D., and Abo,A. (2007). R-Spondin1 regulates Wnt signaling by inhibiting internalization of LRP6. Proc. Natl. Acad. Sci. U. S. A *104*, 14700-14705.

Boman,H.G. (1995). Peptide Antibiotics and their Role in Innate Immunity. Annual Review of Immunology *13*, 61-92.

Breuhahn,K., Singh,S., Schirmacher,P., and Blaker,H. (2008). Large-scale N-terminal deletions but not point mutations stabilize beta-catenin in small bowel carcinomas, suggesting divergent molecular pathways of small and large intestinal carcinogenesis. J Pathol. *215*, 300-307.

Cadwell,K., Liu,J.Y., Brown,S.L., Miyoshi,H., Loh,J., Lennerz,J.K., Kishi,C., Kc,W., Carrero,J.A., Hunt,S., Stone,C.D., Brunt,E.M., Xavier,R.J., Sleckman,B.P., Li,E., Mizushima,N., Stappenbeck,T.S., and Virgin,H.W. (2008). A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature *456*, 259-263.

Cantor, R.M., Lange, K., Sinsheimer, J.S. (2010). Prioritizing GWAS results: A review of statistical methods and recommendations for their application. Am J Hum Genet. *86(1),*6-22.

Cario,E. (2005). Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2. Gut *54,* 1182-1193.

Cario,E., Gerken,G., and Podolsky,D.K. (2002). "For whom the bell tolls!" -- innate defence mechanisms and survival strategies of the intestinal epithelium against lumenal pathogens. Z. Gastroenterol. *40*, 983-990.

Carmon,K.S., Gong,X., Lin,Q., Thomas,A., Liu,Q. (2011) R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/{beta}-catenin signaling. Proc Natl Acad Sci U S A *108(28),* 11452-7.

Carlson,E.A. (2004). Mendel's Legacy: The Origin of Classical Genetics. Cold Spring Harbor Laboratory Press, 2004. ISBN 0-87969-675-3

Castelo-Branco,G., Andersson,E.R., Minina,E., Sousa,K.M., Ribeiro,D., Kokubu,C., Imai,K., Prakash,N., Wurst,W., and Arenas,E. (2009). Delayed dopaminergic neuron differentiation in Lrp6 mutant mice. Dev. Dyn. *239(1),* 211-21.

Cattin,A.L., LE,B.J., Barreau,F., Saint-Just,S., Houllier,A., Gonzalez,F.J., Robine,S., Pincon-Raymond,M., Cardot,P., Lacasa,M., and Ribeiro,A. (2009). Hepatocyte Nuclear Factor-4 alpha: a key factor for homeostasis, cell architecture and barrier function of the adult intestinal epithelium. Mol. Cell Biol. *29(23),* 6294-308. Epub 2009 Oct 5.

Chiche,J.D., Cariou,A., Mira,J.P. (2002) Bench-to-bedside review: fulfilling promises of the Human Genome Project. Crit Care. *6*, 212-5.

Chien,A.J., Moore,E.C., Lonsdorf,A.S., Kulikauskas,R.M., Rothberg,B.G., Berger,A.J., Major,M.B., Hwang,S.T., Rimm,D.L., and Moon,R.T. (2009). Activated Wnt/beta-catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. Proc. Natl. Acad. Sci. U. S. A *106*, 1193-1198.

Collins,F.S., Morgan,M., Patrinos,A. (2003). The Human Genome Project: Lessons from Large-Scale Biology, Science *300*, 286-290

Crosnier,C., Stamataki,D., and Lewis,J. (2006). Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat. Rev. Genet. *7*, 349-359.

De Ferrari,G.V., Papassotiropoulos,A., Biechele,T., Wavrant De-Vrieze,F., Avila,M.E., Major,M.B., Myers,A., Saez,K., Henriquez,J.P., Zhao,A., Wollmer,M.A., Nitsch,R.M., Hock,C., Morris,C.M., Hardy,J., and Moon,R.T. (2007). Common genetic variation within the low-density lipoprotein receptor-related protein 6 and late-onset Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A *104*, 9434-9439.

De Lau,W., Barker,N., and Clevers,H. (2007). WNT signaling in the normal intestine and colorectal cancer. Front Biosci. *12*, 471-491.

De Lau,W., Barker,N., Low,T.Y., Koo,B.K., Li,V.S., Teunissen,H., Kujala,P., Haegebarth,A., Peters,P.J., van de Wetering,M., Stange,D.E., van Es,J., Guardavaccaro,D., Schasfoort,R.B., Mohri,Y., Nishimori,K., Mohammed,S., Heck,A.J., and Clevers,H. (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signaling. Nature *doi: 10.1038/nature10337. [Epub ahead of print]*

De Ridder,L., Weersma,R.K., Dijkstra,G., van der,S.G., Benninga,M.A., Nolte,I.M., Taminiau,J.A., Hommes,D.W., and Stokkers,P.C. (2007). Genetic susceptibility has a more important role in pediatric-onset Crohn's disease than in adult-onset Crohn's disease. Inflamm. Bowel. Dis. *13*, 1083-1092.

Deng,J., Xia,W., Miller,S.A., Wen,Y., Wang,H.Y., and Hung,M.C. (2004). Crossregulation of NF-kappaB by the APC/GSK-3beta/beta-catenin pathway. Mol. Carcinog. *39*, 139-146.

Di,L., Srivastava,S., Zhdanova,O., Ding,Y., Li,Z., Wulff,H., Lafaille,M., and Skolnik,E.Y. (2010). Inhibition of the K+ channel KCa3.1 ameliorates T cell-mediated colitis. Proc. Natl. Acad. Sci. U. S. A *107*, 1541-1546.

Didierlaurent,A., Sirard,J.C., Kraehenbuhl,J.P., and Neutra,M.R. (2002). How the gut senses its content. Cell Microbiol. *4*, 61-72.

Doss,M., White,M.R., Tecle,T., Gantz,D., Crouch,E.C., Jung,G., Ruchala,P., Waring,A.J., Lehrer,R.I., and Hartshorn,K.L. (2009). Interactions of alpha-, beta-, and theta-defensins with influenza A virus and surfactant protein D. J Immunol. *182*, 7878-7887.

Duan,Y., Liao,A.P., Kuppireddi,S., Ye,Z., Ciancio,M.J., and Sun,J. (2007). beta-Catenin activity negatively regulates bacteria-induced inflammation. Lab. Invest. *87*, 613-624.

Duchmann,R., May,E., Heike,M., Knolle,P., Neurath,M., and Meyer zum Büschenfelde,K.-H. (1999). T cell specifity and cross reactivity towards enterobacteria, *Bacteroides*, *Bifidobacterium*, and antigens from resident intestinal flora in humans. Gut *44*, 812-818.

Duval, A., Gayet, J., Zhou ,X.P., Iacopetta, B., Thomas, G., et al. (1999) Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. Cancer Res. *59,* 4213–4215.

Eizaguirre,I., Aldazabal,P., Barrena,M.J., Garcia-Arenzana,J.M., Alcorta,M., Ariz,C., Candelas,S., and Tovar,J.A. (1999). Bacterial translocation is favored by the preservation of the ileocecal valve in experimental short bowel with total parenteral nutrition. Eur. J Pediatr. Surg. *9*, 220-223.

Elphick DA, Mahida YR. Paneth cells: their role in innate immunity and inflammatory disease. Gut *54(12),* 1802-9.

Elphick,D., Liddell,S., and Mahida,Y.R. (2008). Impaired luminal processing of human defensin-5 in Crohn's disease: persistence in a complex with chymotrypsinogen and trypsin. Am J Pathol. *172*, 702-713.

Elston RC, Forthofer R (1977) Testing for Hardy-Weinberg equilibrium in small samples. Biometrics 33, 536-542.

Ericksen,B., Wu,Z., Lu,W., and Lehrer,R.I. (2005). Antibacterial Activity and Specificity of the Six Human alpha-Defensins. Antimicrob. Agents Chemother. *49*, 269-275.

Esche,C., Stellato,C., and Beck,L.A. (2005). Chemokines: key players in innate and adaptive immunity. J Invest Dermatol. *125*, 615-628.

Fedtke,I., Götz,F., Peschel,A. (2004) Bacterial evasion of innate host defences--the Staphylococcus aureus lesson. Int J Med Microbiol. 294,189-94.

Fagotto,F., Jho,E., Zeng,L., Kurth,T., Joos,T., Kaufmann,C., and Costantini,F. (1999). Domains of axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization. J Cell Biol. *145*, 741-756.

Fanaro,S., Chierici,R., Guerrini,P., and Vigi,V. (2003). Intestinal microflora in early infancy: composition and development. Acta Paediatr. Suppl *91*, 48-55.

Fellermann,K., Stange,D.E., Schaeffeler,E., Schmalzl,H., Wehkamp,J., Bevins,C.L., Reinisch,W., Teml,A., Schwab,M., Lichter,P., Radlwimmer,B., and Stange,E.F. (2006). A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. Am J Hum Genet *79*, 439-448.

Fowler,E.V., Doecke,J., Simms,L.A., Zhao,Z.Z., Webb,P.M., Hayward,N.K., Whiteman,D.C., Florin,T.H., Montgomery,G.W., Cavanaugh,J.A., and Radford-Smith,G.L. (2008). ATG16L1 T300A shows strong associations with disease subgroups in a large Australian IBD population: further support for significant disease heterogeneity. Am. J. Gastroenterol. *103*, 2519-2526.

Fox,J.G., Feng,Y., Theve,E.J., Fry,R.C., Fiala,J.L., Doernte,A.L., Williams,M., Raczynski,A.R., McFaline,J.L., Essigmann,J.M., Schauer,D.B., Tannenbaum,S.R., Dedon,P.C., Weinman,S.A., Lemon,S.M., and Rogers,A.B. (2009). Gut microbes define liver cancer risk in mice exposed to chemical and viral transgenic hepatocarcinogens. Gut.

Fre,S., Huyghe,M., Mourikis,P., Robine,S., Louvard,D., and rtavanis-Tsakonas,S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. Nature *435*, 964-968.

Fre,S., Pallavi,S.K., Huyghe,M., Lae,M., Janssen,K.P., Robine,S., rtavanis-Tsakonas,S., and Louvard,D. (2009). Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proc. Natl. Acad. Sci. U. S. A *106*, 6309-6314.

Frei,P., Fried,M., Hungerbuhler,V., Rammert,C., Rousson,V., and Kullak-Ublick,G.A. (2006). Analysis of risk factors for low bone mineral density in inflammatory bowel disease. Digestion *73*, 40-46.

Friis-Hansen,L. (2006). Achlorhydria is associated with gastric microbial overgrowth and development of cancer: lessons learned from the gastrin knockout mouse. Scand. J Clin Lab Invest *66*, 607-621.

Garcia,M.I., Ghiani,M., Lefort,A., Libert,F., Strollo,S., and Vassart,G. (2009). LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. Dev. Biol. *331*, 58-67.

Gasche,C. and Grundtner,P. (2005). Genotypes and phenotypes in Crohn's disease: do they help in clinical management? Gut *54*, 162-167.

George,M.D., Wehkamp,J., Kays,R.J., Leutenegger,C.M., Sabir,S., Grishina,I., Dandekar,S., and Bevins,C.L. (2008). In vivo gene expression profiling of human intestinal epithelial cells: analysis by laser microdissection of formalin fixed tissues. BMC. Genomics *9*, 209.

Gibbs, J.R., Singleton, A., 2006 Application of Genome-Wide Single Nucleotide Polymorphism Typing: Simple Association and Beyond. PLoS Genet *2(10)*, e150. doi:10.1371/journal.pgen.0020150

Gibson, G. (2006). Hints of hidden heritability in GWAS. Nat Genet. *42(7)*, 558-60.

Gill,S.R., Pop,M., Deboy,R.T., Eckburg,P.B., Turnbaugh,P.J., Samuel,B.S., Gordon,J.I., Relman,D.A., Fraser-Liggett,C.M., and Nelson,K.E. (2006). Metagenomic analysis of the human distal gut microbiome. Science *312*, 1355-1359.

Girardin,S.E. (2003). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. Biol Chem *278*, 8869-8872.

Gordon,M.D. and Nusse,R. (2006). Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J. Biol. Chem *281*, 22429-22433.

Gore,R.M. (1997). Small bowel cancer. Clinical and pathologic features. Radiol. Clin North Am. *35*, 351-360.

Grant, S.F., Thorleifsson, G., Reynisdottir, I., Benediktsson, R., Manolescu, A., et al. (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet. *38,* 320–323.

Gregorieff,A., Stange,D.E., Kujala,P., Begthel,H., van den,B.M., Korving,J., Peters,P.J., and Clevers,H. (2009). The Ets-Domain Transcription Factor Spdef Promotes Maturation of Goblet and Paneth Cells in the Intestinal Epithelium. Gastroenterology. *137(4)*,1333-45

Gregorieff,A. and Clevers,H. (2005). Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev. *19*, 877-890.

Haines,J.L., Schnetz-Boutaud,N., Schmidt,S., Scott,W.K., Agarwal,A., Postel,E.A., Olson,L., Kenealy,S.J., Hauser,M., Gilbert,J.R., and Pericak-Vance,M.A. (2006). Functional candidate genes in age-related macular degeneration: significant association with VEGF, VLDLR, and LRP6. Invest Ophthalmol. Vis. Sci. *47*, 329-335.

Halfvarson,J., Jess,T., Magnuson,A., Montgomery,S.M., Orholm,M., Tysk,C., Binder,V., and Jarnerot,G. (2006). Environmental factors in inflammatory bowel disease: a co-twin control study of a Swedish-Danish twin population. Inflamm. Bowel. Dis. *12*, 925-933.

Hampe,J., Franke,A., Rosenstiel,P., Till,A., Teuber,M., Huse,K., Albrecht,M., Mayr,G., De,L., V, Briggs,J., Gunther,S., Prescott,N.J., Onnie,C.M., Hasler,R., Sipos,B., Folsch,U.R., Lengauer,T., Platzer,M., Mathew,C.G., Krawczak,M., and Schreiber,S. (2007). A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet. *39*, 207-211.

Heitman,D.W., Pool,T.B., and Cameron,I.L. (1980). Changes in proliferation and surface morphology in the rat ileum in response to total parenteral nutrition. J Anat. *130*, 603-615.

Hill,W.G., Robertson,A. (1968) The effects of inbreeding at loci with heterozygote advantage Genetics. *60*,615-28.

Hino,S., Michiue,T., Asashima,M., and Kikuchi,A. (2003). Casein kinase I epsilon enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of beta-catenin. J. Biol. Chem *278*, 14066-14073.

Hoffman,J., Kuhnert,F., Davis,C.R., and Kuo,C.J. (2004). Wnts as essential growth factors for the adult small intestine and colon. Cell Cycle *3*, 554-557.

Holmen,S.L., Salic,A., Zylstra,C.R., Kirschner,M.W., and Williams,B.O. (2002). A novel set of Wnt-Frizzled fusion proteins identifies receptor components that activate beta -catenin-dependent signaling. J Biol. Chem. *277*, 34727-34735.

Hugot,J.P., Chamaillard,C., Zouali,H., Lesage,S., Cezard,J.-P., Belaiche,J., Almer,S., Tysk,C., O´Morain,C.A., Gassull,M., Binder,V., Finkel,Y., Cortot,A., Modigliani,R., Laurent-Puig,P., Gower-Rousseau,C., Macry,J., Colombel,J.-F., Sahbatou,M., and Thomas,G. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn´s disease. Nature *411*, 599-603.

Hugot J.P., Laurent-Puig P., Gower-Rousseau C., Olson J.M., Lee J.C., Beaugerie L., Naom I., Dupas J.L., Van Gossum A., Orholm M., Bonaiti-Pellie C., Weissenbach J., Mathew C.G., Lennard-Jones J.E., Cortot A., Colombel J.F., Thomas G.. (1996). Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature *379*, 821-3.

Hung,K.H., Wu,J.J., Yang,H.B., Su,L.J., and Sheu,B.S. (2009). Host Wnt/beta-catenin pathway triggered by Helicobacter pylori correlates with regression of gastric intestinal metaplasia after H. pylori eradication. J Med. Microbiol. *58*, 567-576.

Husebye,E. (2005). The pathogenesis of gastrointestinal bacterial overgrowth. Chemotherapy *51 Suppl 1*, 1-22.

Imielinski M, Baldassano RN, Griffiths A et al. 2009 Common variants at five new loci associated with early-onset inflammatory bowel disease. Nat Genet *2041(12)*, 1335-40.

Innan,H., Stephan,W. (2003) Distinguishing the hitchhiking and background selection models. Genetics 165,2307-12.

Ioannidis,J.P., Castaldi,P., Evangelou,E. (2010). A compendium of genome-wide associations for cancer: critical synopsis and reappraisal. J Natl Cancer Inst. 102,846-58.

Janeway,C. (1989). Immunogenicity signals 1,2,3 ... and 0. Immunol. Today *10*, 283-286.

Janeway,C.A., Jr. (2001). How the immune system protects the host from infection. Microbes. Infect. *3*, 1167-1171.

Janssens, A.C., van Duijn, C.M. (2008). Genome-based prediction of common diseases: advances and prospects. Hum Mol Genet. *17*, 166-73.

Jensen,J., Pedersen,E.E., Galante,P., Hald,J., Heller,R.S., Ishibashi,M., Kageyama,R., Guillemot,F., Serup,P., and Madsen,O.D. (2000). Control of endodermal endocrine development by Hes-1. Nat. Genet. *24*, 36-44.

Jones,D.E. and Bevins,C.L. (1992). Paneth cells of the human small intestine express an antimicrobial peptide gene. J. Biol. Chem. *267*, 23216-23225.

Jorde,L.B. (2000). Linkage disequilibrium and the search for complex disease genes.
Genome Res. *10*, 1435-44.

Kaler,P., Godasi,B.N., Augenlicht,L., and Klampfer,L. (2009). The NF-kappaB/AKT-dependent Induction of Wnt Signaling in Colon Cancer Cells by Macrophages and IL-1beta. Cancer Microenviron. *2(1)*, 69–80.

Kapel,N., Benahmed,N., Morali,A., Svahn,J., Canioni,D., Goulet,O., and Ruemmele,F.M. (2009). Fecal beta-defensin-2 in children with inflammatory bowel diseases. J Pediatr. Gastroenterol. Nutr. *48*, 117-120.

Kaser,A., Lee,A.H., Franke,A., Glickman,J.N., Zeissig,S., Tilg,H., Nieuwenhuis,E.E., Higgins,D.E., Schreiber,S., Glimcher,L.H., and Blumberg,R.S. (2008). XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell *134*, 743-756.

Kim,K.A., Wagle,M., Tran,K., Zhan,X., Dixon,M.A., Liu,S., Gros,D., Korver,W., Yonkovich,S., Tomasevic,N., Binnerts,M., and Abo,A. (2008). R-Spondin family members regulate the Wnt pathway by a common mechanism. Mol. Biol. Cell *19*, 2588-2596.

Klotman,M.E. and Chang,T.L. (2006). Defensins in innate antiviral immunity. Nat Rev Immunol *6*, 447-456.

Kobayashi,K.S., Chamaillard,M., Ogura,Y., Henegariu,O., Inohara,N., Nunez,G., and Flavell,R.A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science *307*, 731-734.

Koch,S., Nava,P., Addis,C., Kim,W., Denning,T.L., Li,L., Parkos,C.A., and Nusrat,A. (2011). The wnt antagonist dkk1 regulates intestinal epithelial homeostasis and wound repair. Gastroenterology *141(1),*259-268.

Kokubu,C., Heinzmann,U., Kokubu,T., Sakai,N., Kubota,T., Kawai,M., Wahl,M.B., Galceran,J., Grosschedl,R., Ozono,K., and Imai,K. (2004). Skeletal defects in ringelschwanz mutant mice reveal that Lrp6 is required for proper somitogenesis and osteogenesis. Development *131*, 5469-5480.

Konigshoff,M., Balsara,N., Pfaff,E.M., Kramer,M., Chrobak,I., Seeger,W., and Eickelberg,O. (2008). Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One. *3*, e2142.

Koprivnjak,T., Peschel,A. (2011). Bacterial resistance mechanisms against host defence peptides. Cell Mol Life Sci. 68, 2243-54.

Korinek,V., Barker,N., Moerer,P., van,D.E., Huls,G., Peters,P.J., and Clevers,H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat. Genet. *19*, 379-383.

Koslowski,M.J., Beisner,J., Stange,E.F., and Wehkamp,J. (2009). Innate antimicrobial host defence in small intestinal Crohn's disease. Int. J Med. Microbiol. *300(1)*, 34-40.

Kübler,I., Koslowski,M.J., Gersemann,M., Fellermann,K., Beisner,J., Becker,S., Rothfuss,K., Herrlinger,K.R., Stange,E.F., and Wehkamp,J. (2009). Influence of standard treatment on ileal and colonic antimicrobial defensin expression in active Crohn's disease. Aliment. Pharmacol Ther. *30(6)*, 621-33

Kübler, Irmgard. Die Rolle von Wnt/Tcf-4 bei Morbus Crohn des Dünndarms. Dissertation. (2007). Inauguraldissertation zur Erlangung des Doktorgrades der Medizin, Medizinische Fakultät der Eberhard-Karls-Universität zu Tübingen.

Kwon,C., Cheng,P., King,I.N., Andersen,P., Shenje,L., Nigam,V., Srivastava,D. 2011. Notch post-translationally regulates β-catenin protein in stem and progenitor cells. Nat Cell Biol. *2011 Aug 14 [Epub ahead of print]*

Lakatos,P.L. (2006). Recent trends in the epidemiology of inflammatory bowel diseases: up or down? World J Gastroenterol. *12*, 6102-6108.

Lala,S., Ogura,Y., Osborne,C., Hor,S.Y., Bromfield,A., Davies,S., Ogunbiyi,O., Nunez,G., and Keshav,S. (2003). Crohn's disease and the NOD2 gene: a role for paneth cells. Gastroenterology *125*, 47-57.

Larsen,S., Bendtzen,K., and Nielsen,O.H. (2010). Extraintestinal manifestations of inflammatory bowel disease: epidemiology, diagnosis, and management. Ann. Med. *42*, 97-114.

Le,B.N., Heresbach,D., Kerbaol,M., Caulet,S., Bretagne,J.F., Chaperon,J., Gosselin,M., and Ramee,M.P. (1995). Histological discrimination of idiopathic inflammatory bowel disease from other types of colitis. J Clin Pathol. *48*, 749-753.

Lees C.W., Barrett J.C., Parkes M., Satsangi J. (2011) New IBD genetics: common pathways with other diseases. *Gut. 2011 Feb 7. [Epub ahead of print]*

Lee,Y.N., Gao,Y., and Wang,H.Y. (2008). Differential mediation of the Wnt canonical pathway by mammalian Dishevelleds-1, -2, and -3. Cell Signal. *20*, 443-452.

Lehrer,R.I., Jung,G., Ruchala,P., Andre,S., Gabius,H.J., and Lu,W. (2009). Multivalent binding of carbohydrates by the human alpha-defensin, HD5. J Immunol. *183*, 480-490.

Lemann,M., Mary,J.Y., Duclos,B., Veyrac,M., Dupas,J.L., Delchier,J.C., Laharie,D., Moreau,J., Cadiot,G., Picon,L., Bourreille,A., Sobahni,I., and Colombel,J.F. (2006). Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. Gastroenterology *130*, 1054-1061.

Levasseur,R., Lacombe,D., and de Vernejoul,M.C. (2005). LRP5 mutations in osteoporosis-pseudoglioma syndrome and high-bone-mass disorders. Joint Bone Spine *72*, 207-214.

Levine,A.D. and Fiocchi,C. (2000). Immunology of inflammatory bowel disease. Curr. Opin Gastroenterol. *16*, 306-309.

Lewontin,R.C. (1964). The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models. Genetics.49,49-67.

Ley,R.E., Peterson,D.A., and Gordon,J.I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell *124*, 837-848.

Lievin-Le,M., V and Servin,A.L. (2006). The front line of enteric host defence against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev *19*, 315-337.

Lindvall,C., Zylstra,C.R., Evans,N., West,R.A., Dykema,K., Furge,K.A., and Williams,B.O. (2009). The Wnt co-receptor Lrp6 is required for normal mouse mammary gland development. PLoS One *4*, e5813.

Liu,G., Bafico,A., Harris,V.K., and Aaronson,S.A. (2003). A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. Mol. Cell Biol. *23*, 5825-5835.

Liu,W., Mani,S., Davis,N.R., Sarrafzadegan,N., Kavathas,P.B., and Mani,A. (2008). Mutation in EGFP domain of LDL receptor-related protein 6 impairs cellular LDL clearance. Circ. Res. *103*, 1280-1288.

Liu,X., Lu,R., Wu,S., Zhang,Y.G., Xia,Y., Sartor,R.B., and Sun,J. (2011). Wnt2 inhibits enteric bacterial-induced inflammation in intestinal epithelial cells. Inflamm Bowel Dis. *2011 Jun 14. [Epub ahead of print]*

Lorentz CP, Wieben ED, Tefferi A, Whiteman DA, Dewald GW. (2002) Primer on medical genomics part I: History of genetics and sequencing of the human genome. Mayo Clin Proc. *77*, 773-82.

Louis,E., Collard,A., Oger,A.F., Degroote,E., Aboul Nasr El Yafi FA, and Belaiche,J. (2001). Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. Gut *49*, 777-782.

MacPherson,A., Khoo,U.Y., Forgacs,I., Philpott-Howard,J., and Bjarnason,I. (1996). Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. Gut *38*, 365-375.

Mahida YR, Rose F, Chan WC. Antimicrobial peptides in the gastrointestinal tract. Gut 1997;40(2):161-3.

Mani,A., Radhakrishnan,J., Wang,H., Mani,A., Mani,M.A., Nelson-Williams,C., Carew,K.S., Mane,S., Najmabadi,H., Wu,D., and Lifton,R.P. (2007). LRP6 mutation in a family with early coronary disease and metabolic risk factors. Science *315*, 1278-1282.

Manicassamy,S., Reizis,B., Ravindran,R., Nakaya,H., Salazar-Gonzalez,R.,M. Wang,Y.-c., Pulendran,B. (2010). Activation of β-catenin in dendritic cells regulates immunity versus tolerance in the intestine. Science *329*, 849–853.

Mao,B., Wu,W., Li,Y., Hoppe,D., Stannek,P., Glinka,A., and Niehrs,C. (2001a). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature *411*, 321-325.

Mao,J., Wang,J., Liu,B., Pan,W., Farr,G.H., III, Flynn,C., Yuan,H., Takada,S., Kimelman,D., Li,L., and Wu,D. (2001b). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol. Cell *7*, 801-809.

Martin,M., Rehani,K., Jope,R.S., and Michalek,S.M. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat. Immunol. *6*, 777-784.

Mastroianni,J.R. and Ouellette,A.J. (2009). alpha-defensins in enteric innate immunity: Functional paneth cell alpha-defensins in mouse colonic lumen. J Biol. Chem.

Mendel, G., 1866, Versuche über Pflanzen-Hybriden. Verh. Naturforsch. Ver. Brünn 4: 3–47 (in English in 1901, J. R. Hortic. Soc. 26, 1–32)

McCarthy, M.I., Abecasis, G.R., Cardon, L.R., Goldstein, D.B., Little, J., Ioannidis, J.P., Hirschhorn, J.N.(2008). Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat Rev Genet. *5, 356-69.*

Medici, V., Mascheretti, S., Croucher, P.J., Stoll, M., Hampe, J., et al. (2006) Extreme heterogeneity in CARD15 and DLG5 Crohn disease-associated polymorphisms between German and Norwegian populations. Eur J Hum Genet *14, 459–468.*

Mondel,M., Schroeder,B.O., Zimmermann,K., Huber,H., Nuding,S., Beisner,J., Fellermann,K., Stange,E.F., and Wehkamp,J. (2008). Probiotic E. coli treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. Mucosal Immunol. *2*, 166-172.

Moore,A.C., Amann,J.M., Williams,C.S., Tahinci,E., Farmer,T.E., Martinez,J.A., Yang,G., Luce,K.S., Lee,E., and Hiebert,S.W. (2008). Myeloid translocation gene family members associate with T-cell factors (TCFs) and influence TCF-dependent transcription. Mol. Cell Biol. *28*, 977-987.

Morgan,T.H. (1916) A Critique of the Theory of Evolution – Chapter III The factorial theory of heredity and the composition of the germplasm

Muise,A.M., Walters,T.D., Glowacka,W.K., Griffiths,A.M., Ngan,B.Y., Lan,H., Xu,W., Silverberg,M.S., and Rotin,D. (2009). Polymorphisms in E-cadherin (CDH1) result in a mis-localised cytoplasmic protein that is associated with Crohn's disease. Gut *58*, 1121-1127.

Naylor,G. and Axon,A. (2003). Role of bacterial overgrowth in the stomach as an additional risk factor for gastritis. Can. J Gastroenterol. *17 Suppl B*, 13B-17B.

Nechiporuk,T., Fernandez,T.E., and Vasioukhin,V. (2007). Failure of epithelial tube maintenance causes hydrocephalus and renal cysts in Dlg5-/- mice. Dev. Cell *13*, 338-350.

Nuding,S., Fellermann,K., Wehkamp,J., and Stange,E.F. (2007). Reduced mucosal antimicrobial activity in Crohn's disease of the colon. Gut *56*, 1240-1247.

Nuding,S., Zabel,L.T., Enders,C., Porter,E., Fellermann,K., Wehkamp,J., Mueller,H.A., and Stange,E.F. (2009). Antibacterial activity of human defensins on anaerobic intestinal bacterial species: a major role of HBD-3. Microbes. Infect. *11*, 384-393.

Odze,R. (2003). Diagnostic problems and advances in inflammatory bowel disease. Mod. Pathol. *16*, 347-358.

Offit,K. (2011) Personalized medicine: new genomics, old lessons Hum Genet. 130,3–14

Ogura,Y., Bonen,D.K., Inohara,N., Nicolae,D.L., Chen,F.F., Ramos,R., Britton,H., Moran,T., Karalluskas,R., Duerr,R.H., Achkar,J.-P., Brant,S.R., Bayless,T.M., Kirschner,B.S., Hanauer,S.B., Nunez,G., and Cho,J.H. (2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn´s diease. Nature *411*, 603-606.

Ogushi,K.F., Wada,A.F., Niidome,T.F., Mori,N.F., Oishi,K.F., Nagatake,T.F., Takahashi,A.F., Asakura,H.F., Makino S FAU - Hojo, Hojo,H.F., Nakahara,Y.F., Ohsaki,M.F., Hatakeyama,T.F., Aoyagi,H.F.,

Kurazono,H.F., Moss,J.F., and Hirayama,T. (2001). Salmonella enteritidis FliC (flagella filament protein) induces hu beta-defensin-2 mRNA production by Caco-2 cells. J. Biol. Chem. *276*, 30521-30526.

Ooi,C.H., Ivanova,T., Wu,J., Lee,M., Tan,I.B., Tao,J., Ward,L., Koo,J.H., Gopalakrishnan,V., Zhu,Y., Cheng,L.L., Lee,J., Rha,S.Y., Chung,H.C., Ganesan,K., So,J., Soo,K.C., Lim,D., Chan,W.H., Wong,W.K., Bowtell,D., Yeoh,K.G., Grabsch,H., Boussioutas,A., and Tan,P. (2009). Oncogenic pathway combinations predict clinical prognosis in gastric cancer. PLoS Genet. *5*, e1000676.

Orford,K., Crockett,C., Jensen,J.P., Weissman,A.M., and Byers,S.W. (1997). Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J. Biol. Chem *272*, 24735-24738.

Otte,J.M., Kiehne,K., and Herzig,K.H. (2003). Antimicrobial peptides in innate immunity of the human intestine. J. Gastroenterol *38*, 717-726.

Ouellette,A.J. (2004). Defensin-mediated innate immunity in the small intestine. Best. Pract. Res Clin Gastroenterol *18*, 405-419.

O´Neil,D.A., Porter,E.M., Elewaut,D., Anderson,G.M., Eckmann,L., Ganz,T., and Kagnoff,M.F. (1999). Expression and regulation of the human □-defensins hBD-1 and hBD-2 in intestinal epithelium. J. Immunol. *163*, 6718-6724.

Papo,N. and Shai,Y. (2005). Host defence peptides as new weapons in cancer treatment. Cell Mol. Life Sci. *62*, 784-790.

Peschel,A. (2002). How do bacteria resist human antimicrobial peptides? Trends Microbiol *10*, 179-186.

Peyrin-Biroulet L, Beisner J, Wang G, Nuding S, Oommen ST, Kelly D, Parmentier-Decrucq E, Dessein R, Merour E, Chavatte P, Grandjean T, Bressenot A, Desreumaux P, Colombel JF, Desvergne B, Stange EF, Wehkamp J, Chamaillard, M..Peroxisome proliferator-activated receptor gamma activation is required for maintenance of innate antimicrobial immunity in the colon. Proc Natl Acad Sci U S A *107*, 8772-7.

Piao,S., Lee,S.H., Kim,H., Yum,S., Stamos,J.L., Xu,Y., Lee,S.J., Lee,J., Oh,S., Han,J.K., Park,B.J., Weis,W.I., and Ha,N.C. (2008). Direct inhibition of GSK3beta by the phosphorylated cytoplasmic domain of LRP6 in Wnt/beta-catenin signaling. PLoS One *3*, e4046.

Pinson,K.I., Brennan,J., Monkley,S., Avery,B.J., and Skarnes,W.C. (2000). An LDL-receptor-related protein mediates Wnt signaling in mice. Nature *407*, 535-538.

Podolsky,D.K. (2002). Inflammatory bowel disease. N. Engl. J. Med. *347*, 417-429.

Rodriguez-Reyna,T.S., Martinez-Reyes,C., and Yamamoto-Furusho,J.K. (2009). Rheumatic manifestations of inflammatory bowel disease. World J Gastroenterol. *15*, 5517-5524.

Rumio,C., Besusso,D., Palazzo,M., Selleri,S., Sfondrini,L., Dubini,F., Menard,S., and Balsari,A. (2004). Degranulation of paneth cells via toll-like receptor 9. Am. J. Pathol *165*, 373-381.

Salzman,N.H., Ghosh,D., Huttner,K.M., Paterson,Y., and Bevins,C.L. (2003). Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature *422*, 522-526.

Salzman,N.H., Hung,K., Haribhai,D., Chu,H., Karlsson-Sjoberg,J., Amir,E., Teggatz,P., Barman,M., Hayward,M., Eastwood,D., Stoel,M., Zhou,Y., Sodergren,E., Weinstock,G.M., Bevins,C.L., Williams,C.B., and Bos,N.A. (2009). Enteric defensins are essential regulators of intestinal microbial ecology. Nat. Immunol. *11*, 76-83.

Sagiv-Friedgut K, Karban A, Weiss B et al. Early-onset Crohn disease is associated with male sex and a polymorphism in the IL-6 promoter. J Pediatr Gastroenterol Nutr. *50*, 22-6.

Sartor,R.B. (2001). Intestinal microflora in human and experimental inflammatory bowel disease. Curr. Opin Gastroenterol. *17*, 324-330.

Sartor,R.B. (2011). Key questions to guide a better understanding of host-commensal microbiota interactions in intestinal inflammation. Mucosal Immunol. *4*, 127-32.

Sarzani,R., Salvi,F., Bordicchia,M., Guerra,F., Battistoni,I., Pagliariccio,G., Carbonari,L., ssi-Fulgheri,P., and Rappelli,A. (2009). Carotid artery atherosclerosis in hypertensive patients with a functional LDL receptor-related protein 6 gene variant. Nutr. Metab Cardiovasc. Dis. *21*, 150-6.

Saulnier,D.M., Kolida,S., and Gibson,G.R. (2009). Microbiology of the human intestinal tract and approaches for its dietary modulation. Curr. Pharm. Des *15*, 1403-1414.

Scherr,R., Essers,J., Hakonarson,H., and Kugathasan,S. (2009). Genetic determinants of pediatric inflammatory bowel disease: is age of onset genetically determined? Dig. Dis. *27*, 236-239.

Schlee,M., Harder,J., Koten,B., Stange,E.F., Wehkamp,J., and Fellermann,K. (2008). Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. Clin Exp Immunol. *151*, 528-535.

Schlee,M., Wehkamp,J., Altenhoefer,A., Oelschlaeger,T.A., Stange,E.F., and Fellermann,K. (2007). The Induction of Human Beta-Defensin-2 by the Probiotic Escherichia coli Nissle 1917 is mediated through Flagellin. Infect Immun. *75*, 2399-407.

Schreiber,S., Rosenstiel,P., Albrecht,M., Hampe,J., and Krawczak,M. (2005). Genetics of Crohn disease, an archetypal inflammatory barrier disease. Nat. Rev. Genet. *6*, 376-388.

Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF, Wehkamp J..Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1. Nature *469*, 419-23.

Schröder,J.M. (1999). Epithelial peptide antibiotics. Biochem. Pharmacol. *57*, 121-134.

Schultz,M. and Lindstrom,A.L. (2008). Rationale for probiotic treatment strategies in inflammatory bowel disease. Expert Rev. Gastroenterol. Hepatol. *2*, 337-355.

Sears, C.L. (2005). A dynamic partnership: celebrating our gut flora, Anaerobe *11*, 247-251

Segditsas,S. and Tomlinson,I. (2006). Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene *25*, 7531-7537.

Semenov,M.V., Tamai,K., Brott,B.K., Kuhl,M., Sokol,S., and He,X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr. Biol. *11*, 951-961.

Setoyama H, Ido A, Numata M, Moriuchi A, Yamaji N, Tamai T, Funakawa K, Fujita H, Sakiyama T, Uto H, Oketani M, Tsubouchi H. (2011) Repeated enemas with hepatocyte growth factor selectively stimulate epithelial cell proliferation of injured mucosa in rats with experimental colitis. Life Sci. *2011 Jul 6. [Epub ahead of print]*

Shivananda,S., Lennard-Jones,J., Logan,R., Fear,N., Price,A., Carpenter,L., and van,B.M. (1996). Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). Gut *39*, 690-697.

Silverberg,M.S., Satsangi,J., Ahmad,T., Arnott,I.D., Bernstein,C.N., Brant,S.R., Caprilli,R., Colombel,J.F., Gasche,C., Geboes,K., Jewell,D.P., Karban,A., Loftus Jr,E.V., Pena,A.S., Riddell,R.H., Sachar,D.B., Schreiber,S., Steinhart,A.H., Targan,S.R., Vermeire,S., and Warren,B.F. (2005). Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can. J Gastroenterol. *19 Suppl A:5-36.*, 5-36.

Simms,L.A., Doecke,J.D., Roberts,R.L., Fowler,E.V., Zhao,Z.Z., McGuckin,M.A., Huang,N., Hayward,N.K., Webb,P.M., Whiteman,D.C., Cavanaugh,J.A., McCallum,R., Florin,T.H., Barclay,M.L., Gearry,R.B., Merriman,T.R., Montgomery,G.W., and Radford-Smith,G.L. (2010). KCNN4 Gene Variant Is Associated With Ileal Crohn's Disease in the Australian and New Zealand Population. Am. J Gastroenterol. *105*, 2209-17.

Simms,L.A., Doecke,J.D., Walsh,M.D., Huang,N., Fowler,E.V., and Radford-Smith,G.L. (2008). Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. Gut *57*, 903-910.

Sims,A.M., Shephard,N., Carter,K., Doan,T., Dowling,A., Duncan,E.L., Eisman,J., Jones,G., Nicholson,G., Prince,R., Seeman,E., Thomas,G., Wass,J.A., and Brown,M.A. (2008). Genetic analyses in a sample of individuals with high or low BMD shows association with multiple Wnt pathway genes. J Bone Miner. Res. *23*, 499-506.

Sirota,M., Schaub,M.A., Batzoglou,S., Robinson,W.H., Butte,A.J. (2009) Autoimmune disease classification by inverse association with SNP alleles. PLoS Genet. 5(12):e1000792.

Song,L., Li,Y., Wang,K., Wang,Y.Z., Molotkov,A., Gao,L., Zhao,T., Yamagami,T., Wang,Y., Gan,Q., Pleasure,D.E., and Zhou,C.J. (2009). Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. Development *136, 3161-3171.*

Spencer,C.C., Deloukas,P., Hunt,S., Mullikin,J., Myers,S., Silverman,B., Donnelly,P., Bentley,D., McVean,G. (2006) The influence of recombination on human genetic diversity. PLoS Genet. *2*, e148.

Stange, E.F. (2009) Colitis ulcerose - Morbus Crohn. 3. Auflage UNI-MED, Bremen ISBN-10: 3837411591; ISBN-13: 9783837411591

Stange,E.F., Travis,S.P., Vermeire,S., Beglinger,C., Kupcinkas,L., Geboes,K., Barakauskiene,A., Villanacci,V., Von,H.A., Warren,B.F., Gasche,C., Tilg,H., Schreiber,S.W., Scholmerich,J., and Reinisch,W. (2006). European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. Gut *55 Suppl 1*, i1-15.

Steenwinckel,V., Louahed,J., Lemaire,M.M., Sommereyns,C., Warnier,G., McKenzie,A., Brombacher,F., Van,S.J., and Renauld,J.C. (2009). IL-9 promotes IL-13-dependent paneth cell hyperplasia and up-regulation of innate immunity mediators in intestinal mucosa. J Immunol. *182*, 4737-4743.

Stoll,M., Corneliussen,B., Costello,C.M., Waetzig,G.H., Mellgard,B., Koch,W.A., Rosenstiel,P., Albrecht,M., Croucher,P.J., Seegert,D., Nikolaus,S., Hampe,J., Lengauer,T., Pierrou,S., Foelsch,U.R., Mathew,C.G., Lagerstrom-Fermer,M., and Schreiber,S. (2004). Genetic variation in DLG5 is associated with inflammatory bowel disease. Nat. Genet. *36*, 476-480.

Strober,W. (2006). Immunology. Unraveling gut inflammation. Science. *313*, 1052-1054.

Takano-Maruyama,M., Hase,K., Fukamachi,H., Kato,Y., Koseki,H., and Ohno,H. (2006). Foxl1-deficient mice exhibit aberrant epithelial cell positioning resulting from dysregulated EphB/EphrinB expression in the small intestine. Am. J Physiol Gastrointest. Liver Physiol *291*, G163-G170.

Tamai,K., Semenov,M., Kato,Y., Spokony,R., Liu,C., Katsuyama,Y., Hess,F., Saint-Jeannet,J.P., and He,X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature *407*, 530-535.

Tanabe,H., Ayabe,T., Bainbridge,B., Guina,T., Ernst,R.K., Darveau,R.P., Miller,S.I., and Ouellette,A.J. (2005). Mouse Paneth Cell Secretory Responses to Cell Surface Glycolipids of Virulent and Attenuated Pathogenic Bacteria. Infect. Immun. *73*, 2312-2320.

Tenesa,A., Noble, C., Satsangi, J., and Dunlop, M. (2006) Association of DLG5 and inflammatory bowel disease across populations. Eur J Hum Genet . *14, 259–260*.

The International HapMap Consortium. (2005). A haplotype map of the human genome. Nature 437, 1299-1320.

The International HapMap Consortium. (2007). A second generation human haplotype map of over 3.1 million SNPs. Nature 449, 851-861.

Thompson,G.R. and Trexler,P.C. (1971). Gastrointestinal structure and function in germ-free or gnotobiotic animals. Gut *12*, 230-235.

Tortora,G.J. Derrickson,B. Principles of Anatomy and Physiology (2008) John Wiley and Sons, Chapter 24, 922-924.

Travis,S.P., Stange,E.F., Lemann,M., Oresland,T., Chowers,Y., Forbes,A., D'Haens,G., Kitis,G., Cortot,A., Prantera,C., Marteau,P., Colombel,J.F., Gionchetti,P., Bouhnik,Y., Tiret,E., Kroesen,J., Starlinger,M., and Mortensen,N.J. (2006). European evidence based consensus on the diagnosis and management of Crohn's disease: current management. Gut *55 Suppl 1*, i16-i35.

Untergasser,A., Nijveen,H., Rao,X., BisselingT., Geurts. R., and Leunissen, J. A.M. (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Research *35*, W71-W74.

Uronis,J.M., Muhlbauer,M., Herfarth,H.H., Rubinas,T.C., Jones,G.S., and Jobin,C. (2009). Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. PLoS One *4*, e6026.

Vaishnava,S., Behrendt,C.L., Ismail,A.S., Eckmann,L., and Hooper,L.V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc. Natl. Acad. Sci. USA *105*, 20858-20863.

van Es,J.H., Jay,P., Gregorieff,A., van Gijn,M.E., Jonkheer,S., Hatzis,P., Thiele,A., van den,B.M., Begthel,H., Brabletz,T., Taketo,M.M., and Clevers,H. (2005). Wnt signaling induces maturation of Paneth cells in intestinal crypts. Nat Cell Biol. *7*, 381-386.

Van Heyningen,V., Yeyati,P.L. (2004). Mechanisms of non Mendelian inheritance in genetic disease. Hum. Mol. Genet. 13 Spec No 2, R225–33.

van Meurs,J.B., Trikalinos,T.A., Ralston,S.H., Balcells,S., Brandi,M.L., Brixen,K., Kiel,D.P., Langdahl,B.L., Lips,P., Ljunggren,O., Lorenc,R., Obermayer-Pietsch,B., Ohlsson,C., Pettersson,U., Reid,D.M., Rousseau,F., Scollen,S., Van,H.W., Agueda,L., Akesson,K., Benevolenskaya,L.I., Ferrari,S.L., Hallmans,G., Hofman,A., Husted,L.B., Kruk,M., Kaptoge,S., Karasik,D., Karlsson,M.K., Lorentzon,M., Masi,L., McGuigan,F.E., Mellstrom,D., Mosekilde,L., Nogues,X., Pols,H.A., Reeve,J., Renner,W., Rivadeneira,F., van Schoor,N.M., Weber,K., Ioannidis,J.P., and Uitterlinden,A.G. (2008). Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. JAMA *299*, 1277-1290.

van-Wetering,S., Sterk,P.J., Rabe,K.F., and Hiemstra,P.S. (1999). Defensins: Key players or bystanders in infection, injury, and repair of the lung. J. Allergy Clin. Immunol. *104*, 1131-1138.

Vidrich,A., Buzan,J.M., Brodrick,B., Ilo,C., Bradley,L., Fendig,K.S., Sturgill,T., and Cohn,S.M. (2009). Fibroblast growth factor receptor-3 regulates Paneth cell lineage allocation and accrual of epithelial stem cells during murine intestinal development. Am. J Physiol Gastrointest. Liver Physiol *297*, G168-G178.

Walker,F., Zhang,H.H., Odorizzi,A., Burgess,A.W. (2011) LGR5 Is a Negative Regulator of Tumourigenicity, Antagonizes Wnt Signaling and Regulates Cell Adhesion in Colorectal Cancer Cell Lines. PLoS One. 6(7):e22733.

Walsh,N.C., Reinwald,S., Manning,C.A., Condon,K.W., Iwata,K., Burr,D.B., and Gravallese,E.M. (2009a). Osteoblast function is compromised at sites of focal bone erosion in inflammatory arthritis. J Bone Miner. Res. *24*, 1572-1585.

Walsh,R., Seth,R., Behnke,J., Potten,C.S., and Mahida,Y.R. (2009b). Epithelial stem cell-related alterations in Trichinella spiralis-infected small intestine. Cell Prolif. *42*, 394-403.

Wang,G., Stange,E.F., and Wehkamp,J. (2007). Host-microbe interaction: mechanisms of defensin deficiency in Crohn's disease. Expert Rev. Anti. Infect. Ther *5*, 1049-1057.

Watson,J.D., Crick,F.H. (1953). Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature *171*, 737–8.

Wehkamp,J., Chu,H., Shen,B., Feathers,R.W., Kays,R.J., Lee,S.K., and Bevins,C.L. (2006). Paneth cell antimicrobial peptides: topographical distribution and quantification in human gastrointestinal tissues. FEBS Lett. *580*, 5344-5350.

Wehkamp,J., Fellermann,K., Herrlinger,K.R., Bevins,C.L., and Stange,E.F. (2005a). Mechanisms of disease: defensins in gastrointestinal diseases. Nat Clin Pract. Gastroenterol Hepatol *2*, 406-415.

Wehkamp,J., Harder,J., Wehkamp,K., Wehkamp-von Meissner,B., Schlee,M., Enders,C., Sonnenborn,U., Nuding,S., Bengmark,S., Fellermann,K., Schroder,J.M., and Stange,E.F. (2004a). NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. Infect. Immun. *72*, 5750-5758.

Wehkamp,J., Harder,J., Weichenthal,M., Mueller,O., Herrlinger,K.R., Fellermann,K., Schroeder,J.M., and Stange,E.F. (2003a). Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. Inflamm. Bowel. Dis. *9*, 215-223.

Wehkamp,J., Harder,J., Weichenthal,M., Schwab,M., Schaffeler,E., Schlee,M., Herrlinger,K.R., Stallmach,A., Noack,F., Fritz,P., Schroder,J.M., Bevins,C.L., Fellermann,K., and Stange,E.F. (2004b). NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut *53*, 1658-1664.

Wehkamp,J., Koslowski,M., Wang,G., and Stange,E.F. (2008). Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease. Mucosal Immunol. *1 Suppl 1*, S67-S74.

Wehkamp,J., Salzman,N.H., Porter,E., Nuding,S., Weichenthal,M., Petras,R.E., Shen,B., Schaeffeler,E., Schwab,M., Linzmeier,R., Feathers,R.W., Chu,H., Lima,H., Jr., Fellermann,K., Ganz,T., Stange,E.F., and Bevins,C.L. (2005b). Reduced Paneth cell alpha-defensins in ileal Crohn's disease. Proc Natl Acad. Sci. U S. A *102*, 18129-18134.

Wehkamp,J., Schmidt,K., Herrlinger,K.R., Baxmann,S., Behling,S., Wohlschlager,C., Feller,A.C., Stange,E.F., and Fellermann,K. (2003b). Defensin pattern in chronic gastritis: HBD-2 is differentially expressed with respect to Helicobacter pylori status. J. Clin. Pathol. *56*, 352-357.

Wehkamp,J., Schwind,B., Herrlinger,K.R., Baxmann,S., Schmidt,K., Duchrow,M., Wohlschlager,C., Feller,A.C., Stange,E.F., and Fellermann,K. (2002). Innate immunity and colonic inflammation: enhanced expression of epithelial alpha-defensins. Dig. Dis. Sci. *47*, 1349-1355.

Wehkamp,J., Wang,G., Kubler,I., Nuding,S., Gregorieff,A., Schnabel,A., Kays,R.J., Fellermann,K., Burk,O., Schwab,M., Clevers,H., Bevins,C.L., and Stange,E.F. (2007). The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. J Immunol. *179*, 3109-3118.

Wheeler,J.M., Warren,B.F., Mortensen,N.J., Kim,H.C., Biddolph,S.C., Elia,G., Beck,N.E., Williams,G.T., Shepherd,N.A., Bateman,A.C., and Bodmer,W.F. (2002). An insight into the genetic pathway of adenocarcinoma of the small intestine. Gut *50*, 218-223.

Willert,K., Brink,M., Wodarz,A., Varmus,H., and Nusse,R. (1997). Casein kinase 2 associates with and phosphorylates dishevelled. EMBO J. *16*, 3089-3096.

Williams,B.O. and Insogna,K.L. (2009). Where Wnts went: the exploding field of Lrp5 and Lrp6 signaling in bone. J Bone Miner. Res. *24*, 171-178.

Wilson,C.L., Ouellette,A.J., Satchell,D.P., Ayabe,T., Lopez-Boado,Y.S., Stratman,J.L., Hultgren,S.J., Matrisian,L.M., and Parks,W.C. (1999). Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defence. Science *286*, 113-117.

Wilson,N.J., Boniface,K., Chan,J.R., McKenzie,B.S., Blumenschein,W.M., Mattson,J.D., Basham,B., Smith,K., Chen,T., Morel,F., Lecron,J.C., Kastelein,R.A., Cua,D.J., McClanahan,T.K., Bowman,E.P., and de Waal,M.R. (2007). Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nat Immunol. *8*, 950-957.

Winton,D.J. and Ponder,B.A. (1990). Stem-cell organization in mouse small intestine. Proc. Biol. Sci. *241*, 13-18.

Witte, J.S. (2010). Genome-wide association studies and beyond. Annu Rev Public Health *31*, 9-20.

Yamanaka,T., Helgeland,L., Farstad,I.N., Fukushima,H., Midtvedt,T., and Brandtzaeg,P. (2003). Microbial colonization drives lymphocyte accumulation and differentiation in the follicle-associated epithelium of Peyer's patches. J Immunol. *170*, 816-822.

Yanagawa,S., van,L.F., Wodarz,A., Klingensmith,J., and Nusse,R. (1995). The dishevelled protein is modified by wingless signaling in Drosophila. Genes Dev. *9*, 1087-1097.

Yang,D., Biragyn,A., Hoover,D.M., Lubkowski,J., and Oppenheim,J.J. (2004). Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defence. Annu. Rev Immunol *22*, 181-215.

Zhao,J., de,V.J., Narushima,S., Beck,E.X., Palencia,S., Shinkawa,P., Kim,K.A., Liu,Y., Levy,M.D., Berg,D.J., Abo,A., and Funk,W.D. (2007). R-spondin1, a novel intestinotrophic mitogen, ameliorates experimental colitis in mice. Gastroenterology *132*, 1331-1343.

Zhao,J., Kim,K.A., de,V.J., Palencia,S., Wagle,M., and Abo,A. (2009). R-Spondin1 protects mice from chemotherapy or radiation-induced oral mucositis through the canonical Wnt/beta-catenin pathway. Proc. Natl. Acad. Sci. USA *106*, 2331-2336.

Zhou,C.J., Pinson,K.I., and Pleasure,S.J. (2004). Severe defects in dorsal thalamic development in low-density lipoprotein receptor-related protein-6 mutants. J Neurosci. *24*, 7632-7639.

Zilbauer M, Jenke A, Wenzel G, Goedde D, Postberg J, Phillips AD, Lucas M, Noble-Jamieson G, Torrente F, Salvestrini C, Heuschkel R, Wirth S. (2010). Intestinal alpha-defensin expression in pediatric inflammatory bowel disease. Inflamm Bowel Dis. *17*, 2076-86.

Zouhairi,M.E., Venner,A., Charabaty,A., and Pishvaian,M.J. (2008). Small bowel adenocarcinoma. Curr. Treat. Options. Oncol. *9*, 388-399.

5. SUMMARY

The human intestinal barrier is constantly challenged by ingested pathogens and a complex commensal flora. To fend off and control the large amount of microbes, the epithelial lining critically depends on innate antimicrobial peptides. Before the major importance of these innate defense molecules became evident, scientists who were investigating Inflammatory Bowel Diseases (IBD) supported an autoimmune pathogenesis concept for the severe reoccurring inflammations in the intestinal tract. More than 10 years ago, when it became evident that gut bacteria form the major target for activated immune cells which underlie the inflammatory symptoms in IBD, the research focus started to shift towards investigations on primary barrier defects. For Crohn's disease (CD), a major form of IBD, different location specific impairments in the epithelial production of antimicrobials have been identified. These not only explain the stability of disease location, but also accommodate an inherited and an environmental component in the disease. In small intestinal CD, such impairments are found in the transcriptional expression and in the storage/secretion of Paneth cell (PC) derived human defensin (HD)-5 and -6. Very recently, the transcription factor TCF7L2 was introduced in the context of their decreased mRNA levels in patients. TCF7L2 is not only a major player in β- catenin dependent Wnt signaling and consequently intestinal epithelial proliferation and Paneth cell maturation, but also directly regulates the transcriptional expression of HD5 and -6.

The main goal of my studies was to further elucidate the primary role of antimicrobial peptides in CD and especially the influence of defective Paneth cell function. This was in large parts done by investigations on underlying genetic mechanisms which affect the Wnt pathway. In 3 DNA sets with together more than 3000 samples, it was possible to elucidate an association of the TCF7L2 putative promotor SNP rs3814570 with ileal CD. In a subsequent approach the Wnt co- receptor LRP6 could also be identified as a new candidate in ileal CD. The hypothesis and literature based candidate gene approach on *LRP6* revealed an association of a coding minor variant, which has a negative effect on the co- receptors function, with early onset and penetrating ileal CD. Since the variant seems to precede further diminished HD5 levels in patients, it might explain reduced antibacterial killing in some affected individuals. More importantly, like the association of *TCF7L2*, it supports the role of primary Paneth cell defects as a major mechanism in the disease. Besides being affected on the genetic level in certain disease subgroups, LRP6 also shows diminished mRNA expression in small intestinal CD in general. This might be an additional and more general mechanism preceding reduced Paneth cell antimicrobial function, but further research is needed to shed light on the exact circumstances. In a parallel approach regarding the role of antimicrobials in active CD and the effect of standard medication on their expression, it was furthermore possible to confirm a lack of coherency between inflammation severity and diminished Paneth cell α- defensins and therefore the primary character of the decrease.

The herein reported results not only help to understand disease underlying mechanisms as they support the concept of defective β- catenin dependent Wnt in ileal CD, but also underline the importance of reduced antimicrobial function in patients.

Additional investigations will be needed to first elucidate a potential role of defective cell differentiation and secondly to identify new targets for therapeutic approaches in the disease. Such new therapeutics would have to strengthen the innate intestinal barrier and therefore, different from current medications, function as a causative treatment.

5. ZUSAMMENFASSUNG

Das Darmepithel eines jeden Menschen muss sich ständiger Bedrohungen durch Pathogene erwehren und eine immense Vielfalt kommensaler Mikroorganismen in Schach halten. Um diesen Angriffen standhalten zu können, ist es auf das angeborene Immunsystem und im speziellen auf antibakterielle Peptide angewiesen. Bevor die große Bedeutung dieser körpereigenen Antibiotika aufgeklärt wurde, wurden Chronisch Entzündliche Darmerkrankungen (CED), schwere wiederkehrenden Entzündungen des Intestinaltrakts, als Autoimmunerkrankung eingestuft. Nachdem jedoch bereits vor über zehn Jahren Darmbakterien als die eigentlichen Auslöser der Symptomatik identifiziert wurden, richteten sich die Forschungsarbeiten auf Barriere-Funktionsstörungen. Für die verschiedenen intestinalen Lokalisationen des Morbus Crohn, einer großen Untergruppe der CED, konnten in Folge dessen spezifische Defekte im System der epithelialen antimikrobiellen Peptide beschrieben werden. Diese Defekte erklären nicht nur das stabile Auftreten der Symptome in definierten Arealen, sondern werden sowohl der erblichen als auch der umweltbedingten Komponente der Krankheit gerecht. Bei Morbus Crohn des Dünndarms gehen Einschränkungen der epithelialen Bakterienabwehr auf Störungen in der Transkription sowie der Speicherung, Abgabe und Aktivierung der Panethzell- „human defensins“ (HD)-5 and -6 zurück. Kürzlich konnte die reduzierte mRNA Expression beider dieser α-Defensine zudem mit verminderten Leveln des Transkriptionsfaktor TCF7L2 in Verbindung gebracht werden. TCF7L2 spielt eine ausschlaggebende Rolle im β- catenin abhängigen Wnt Signalweg und ist folglich wichtig für die epitheliale Proliferation sowie die Panethzellreifung und die transkriptionelle Regulation von HD5 und -6.

Die hierin präsentierten Arbeiten beschäftigen sich mit der weitere Untersuchung der Rolle antimikrobieller Peptide und im Besonderen des Einflusses gestörter Panethzellfunktion bei Morbus Crohn. Das Hauptaugenmerk lag dabei in der Identifikation und Aufklärung genetischer Pathogenesemechanismen die den Wnt Signalweg betreffen. In den dafür untersuchten und analysierten großen CED DNA Kohorten, konnte ein im putativen Promotor von *TCF7L2* lokalisierter SNP (rs3814570) mit Morbus Crohn des Dünndarms assoziiert werden. In einem weiteren Studienansatz war es zudem möglich den Wnt Ko-rezeptor LRP6 als einen neuen Faktor in der Erkrankung einzustufen. In einer auf Literaturrecherche basierender und

hypothesengetriebener Kandidatengenstudie, konnte eine funktionelle SNP- Variante mit besonders früh auftretender und penetrierender Symptomatik des Dünndarmcrohns assoziiert werden. Da die Variante potentiell zu einer weiteren Verminderung in der HD5 Transkription führt, erklärt sie somit teilweise die gestörte antibakterielle Funktion einiger Patienten und unterstreicht, wie auch die Assoziation des Kandidatengens *TCF7L2*, die zentrale Rolle der Panethzelle. Neben der genetischen Beeinträchtigung in einigen Untergruppen, weist LRP6 zusätzlich eine allgemein verminderte mRNA Expression im Epithel von Dünndarmcrohnpatienten auf. Dies stellt unter Umständen einen allgemeineren Pathomechanismus dar, der weitere Untersuchungen zur genaueren Aufklärung bedarf. Parallel zu den genetischen Analysen, konnte in einer Untersuchung betreffend antimikrobieller Peptide und deren mögliche Beeinflussung durch Standardmedikation bei aktivem Morbus Crohn, ein fehlender Zusammenhang zwischen der Entzündungsstärke und verminderter HD5 und -6 Expression bestätigt werden.

Die hier zusammengetragenen Ergebnisse unterstützen nicht nur das Konzept eines Wnt- Signalwegdefektes als Pathogenesemechanismus bei Morbus Crohn des Dünndarms, sondern unterstreichen in ihrer Gesamtheit auch die tragende und primäre Rolle reduzierter antimikrobieller Abwehr in Patienten. Weitere Untersuchungen werden nötig sein um eine eventuelle Beteiligung gestörter Zelldifferenzierung aufzuklären und um neue Therapiemöglichkeiten aufzudecken. Solch neue Optionen sollten im Gegensatz zur derzeit verwendeten Standardmedikation gezielt die angeborene Barrierefunktion unterstützen und daher als kausale Therapie fungieren.

APPENDIX

1. LRP6 rs2302685 frequency distribution in the separate cohort samples (a: Leuven, b: Vienna): the different distribution of genotypes is demonstrated for each group and subgroup: controls, ulcerative colitis (UC), Crohn´s Disease (CD) with solely colonic involvement (colonic CD; L2) and CD with solely ileal as well as ileal and colonic involvement (ileal CD; L1+L3). The ileal CD group was further sub grouped according to gender, disease behaviour (inflammatory B1; stricturing B2; penetrating B3) and age at diagnosis (older then 17: >17; 17 and younger: </=17)

		Overview groups				*Overview ileal CD*						
Leuven			**IBD**			**Gender**		**disease behaviour**			**Age at diagnose**	
		controls	*UC*	*colonic CD*	*ileal CD*	*male*	*female*	*B1*	*B2*	**B3**	*>17*	*</= 17*
	TT	194	56	25	137	61	77	27	43	**61**	127	**10**
Genotypes	CT	91	32	13	66	29	40	13	10	**42**	62	**4**
(n)	CC	9	0	1	10	4	6	1	4	**4**	6	**4**
samples (n)	all	294	88	39	213	94	123	41	57	**107**	195	**18**
	TT	65.99	63.64	64.1	64.32	64.89	62.6	65.85	75.44	**57.01**	65.13	**55.56**
Genotypes	CT	30.95	36.36	33.33	30.99	30.85	32.52	31.71	17.54	**39.25**	31.79	**22.22**
(%)	CC	3.06	0	2.56	4.69	4.26	4.88	2.44	7.02	**3.74**	3.08	**22.22**
Alleles	T	81.46	81.82	80.77	79.81	80.32	78.86	81.71	84.21	**76.64**	81.03	**66.67**
(%)	C	18.54	18.18	19.23	20.19	19.68	21.14	18.29	15.79	**23.36**	18.97	**33.33**

		Overview groups				*Overview ileal CD*						
Vienna			**IBD**			**Gender**		**disease behaviour**			**Age at diagnose**	
		controls	*UC*	*colonic CD*	*ileal CD*	*male*	*female*	*B1*	*B2*	*B3*	*>17*	*</= 17*
	TT	614	286	34	125	68	56	38	31	55	118	**7**
Genotypes	CT	277	147	18	74	33	41	27	12	35	64	**10**
(n)	CC	32	14	2	5	3	2	2	1	3	4	**1**
samples (n)	all	923	447	54	204	104	99	67	44	93	186	**18**
	TT	66.52	63.98	62.96	61.27	65.38	56.57	56.72	70.45	59.14	63.44	**38.89**
Genotypes	CT	30.01	32.89	33.33	36.27	31.73	41.41	40.3	27.27	37.63	34.41	**55.56**
(%)	CC	3.47	3.13	3.7	2.45	2.88	2.02	2.99	2.27	3.23	2.15	**5.56**
Allele	T	81.53	80.43	79.63	79.41	81.25	77.27	76.87	84.09	77.96	80.65	**66.67**
Frequency	C	18.47%	19.57%	20.37%	20.59%	18.75%	22.73%	23.13%	15.91 %	22.04 %	19.35	**33.33**

2. Sequences and primer used in the sequencing of *TCF7L2* exonic and intron boundary regions. Sequences originated from Ensemble and primers were designed as stated in the methods part. Ensemble exons are marked via a grey background. Primers are marked (1 font size up and bold) and their location is referenced. Sequences of reverse primers are logically displayed complementary accoring to their sequence. Location of SNPs which were stated in the Ensemble data base are marked white.

Exon 1

TAACTTCAACTCACTCAAATCCGAGCG**GCACGAGCACCTCCTGTATC**[1]TTCGGCTTCCCCCCCCCTTTGCTCTTTATATCTGACT
TCTTGTTGTTGTTGGTGTTTTTTTTTTTTTACCCCCCTTTTTTATTTATTATTTTTTTGCACATTGATCGGATCCTTGGGAACGAGA
GAAAAAAGAAACCCAA**ACTCACGCGTGCAGAAGA**[2]TCTCCCCCCCCTTCCCCTCCCCTCCTCCCTCTTTTCCCCTCCCCAGGAG
AAAAAGACCCCCAAGCAGAAAAAAGTTCACCTTGGACTCGTCTTTTTCTTGCAATATTTTTTGGGGGGGCAAAACTTTTTGGGGG
TGATTTTTTT**TGGCTTTTCTTCCTCCTTCA**[3]TTTTTCTTCCAAAATTGCTGCTGGTGGGTGAAAAAAAAATGCCGCAGCTGAAC
GGCGGTGGAGGGGATGACCTAGGCGCCAACGACGAACTGATTTCCTTCAAAGACGAGGGCGAACAGGAGGAGAAGAGCTCC
GAAAACTCCTC**GGCAGAGAGGGATTTAGCG**[4]ATGTCAAATCGTCTCTAGTCAATGAATCAGAAACGAATCAAAACAGCTCCT
CCGATTCCGAGGTAGGAAAAGCCCCTCGGGCTGGTGGGGTTTTTTATCTGTTTCCTGGG**CTTGGCAAATGTTGCTGAA**[5]AGG
GGAGAAATCGGGGCTGGGGGCGGCGGCGGGGCCCGG

Exon 2/3

CCCCCCTCG**ACCTCGCCGATTCTTTTTCT**[6]CCCCCTTCTCCCCCTTCTGCGTGGCGTTTGCCCCTCGCCCTCCCCACCTCCACCCC
TCTGGGAAGGCGGAAAGACGGCCTCCGCCTCGCTCCGAAAGTTTCCGAGACAAATCCCGGGAAAGTTTGGAAGAAGGTGAGT
ACGCCCCGCGCGCCCCGCAGCCGCCCGGAGCCGCCCCCCGGGCCGGCCGCCCCGCGCGCCCCGGCCCCGCGCCCGGCTCGGCC
TGGCCCTGCGCCGGCCCGGTCGGGGCGCCCGGC**CCCTCGGGGCACTTTCTA**[7]AAAAGTTTC**TCCTCACTCTCTCCCGCTC**[8]
CGCGCGGCCGCCGCTGTCCCCTCGCCGCCCCGCCATGTTAGCGGCCAAGAGGCAAGATGGAGGGCTCTTTAAGGGGCCACCGT
ATCCCGGCTACCCCTTCATCATGATCCCCGACCTGACGAGCCCCTACCTCCCCAACGGATCGCTCTCGCCCACCGCCCGAACCGT
AAGTGCCTCCGCGCCCGGCCCCCGCCCGCTGCCCGCCCGCCCGCGCCGCCCGCCGGGCCCCGCATGCGGCCCCTGCCCTGCCCC
CTCCCCGCCTCCCCCTCCCCGCCTCCTCCCCTCCCTCCCTCCCCTCCCCCGCTCGTGGCC**CAGGAGGCCCGAAACTCT**[9]

Exon 4

TTGATTGAATGCCAA**GGCTGCAGTTCAATCTAGGAA**[10]GGTTTGTCTGCTATTCATAAACTGCTTAAGATGTTTTCTTGTAGTT
TGTGACATAAGCAGAACGCTTTGATTTGGTTTCTTTCTACAGCTCCATTTTCAGTCCGGCAGCACACATTACTCTGCGTACAAAAC
GATTGAACACCAGATTGCAGTTCAGGTAGGAAACGCAAGAGATTCTGAAGCTTGAATTGTCTATATGTAGGTCTCTTGTGTGTTT
TATTCTCCGCCCCTTCCCCCAACTGAGATRATGATGATGATGATGAT |{base14863:A/G}GATGATGATGRTGGTGGTGGTG
GTGGTGATGGTGGTGGTGGTGGTGATGGTGGTGGTGGT |{base14891:G/A}GATGGTGGTGGTGGTGGTGGGGGGG
GG**TTGAATCACTGGGGGAGAAG**[11]GGGAGCAAAG

1 TCF4 E1 A1S1 forward
2 TCF4 E1 S2 forward
3 TCF4 E1 S3 forward
4 TCF4 E1 S4 forward
5 TCF4 E1 A2 reverse
6 TCF4 E2/3 A1/E2S1 forward
7 TCF4 E2/3 E3S1 forward
8 TCF4 E2/3 S2 reverse
9 TCF4 E2/3 A2/E3S2 reverse
10 TCF4 E5neu A1S1 forward
11 TCF4 E5neu A2S2 reverse

Exon 5

TCTGAGGAAGACCCTATTGGAATTGCATGCTTTTTTATTTTTTTAATGATTATTTGCATGCTTGTATGTTT**TTCAGTTTCTGACCC ATGTCAC**[12]AGTTATTTCTTGGGCTAGTTGTTCTGCATTTACTTTCTGAATTCATTGTTTTTCATTTCACTTTTGTTTCCTCTCGCCA GTATCTCCAGATGAAATGGCCACTGCTTGATGTCCAGGCAGGGAGCCTCCAGAGTAGACAAGCCCTCAAGGATGCCCGGTCCC CATCACCGGCACACATTGTCGTAAGTAACCTCCCAGAGATGATGGCTTCCTTTATTGAGGGGGTGAAAAAGAAAATGCTTT**TTT GATGATAACAGGCCTTATTTG**[13]TCATTTTTTTCTTTCTTTAAAC

Exon 6

AACTGACTTCTTTCACTTAGCAAATGGAAGGGTTTTTGATGGAATCTTAGCTCAGATGTCGCCCTAG**GCTTGGCCTTCTGGAA CTCA**[14]CTGTTCTCTTGCTTCCTTCCACCCTGCCTCCCTACCCGCTTTGCTACAAGAAGACAGAATGTAGAAACCCCCAGTTGACTT TTTGAGTAGAAATGAGCCATGTTCTATTTAGTGGTAGAATGTTTGCTGGGTTAGCCCAGGACTTCAGATGGAGTGCTTCCTCCTT TTTAATACCTTGTGACTGCTGTGTTGGCAAGCAGAGGGTTAAATGCTGCAGGAGGACATGGGAGAGTTTTTGCTTTGA**GGGTC CACTGGAGCAAGAGA**[15]AGCTTGAGAGTCTGTCTCTGAGATCYCAGAACAGACATGAAGATATGGTACCGATTCCCTTGGTG CTAA |{base121577:C/T}AAGGAACTCCAGGGGAATTTCAGGAAACCCCTCCTTTTCTCGTCTTTATTCTCACCCCGTCCCCCC GCCACCTCTCTCCCCAAGTTCACACTCAGGAGGGAGGAATCTTTTACCTTTTCCGTTCTGGGCAGCAGCTGTAGGGAATGTTATC AGTGTTGGGACTGGGCACGGGACCAAGCCTTCCTGGTGCCTGGACTGCAACCWAGCTGAGAGCTGACATGGCAGGGCGAGTT GTGTGTG |{base 121824:A/T}AGTTGGCTGCCCCTAGGCTCAT|{base 121877:G/}AGGCCTAGCACAATAGAGAC**CCTG TCAATGGAGGGACACT**[16]ATCAGGCAGGTCACTATCAATGTGACAGACACTATCAGGCAGGTCACATTGGTGCAGA

Exon 7

A**GAAATGTGGGTTAGGGCAGA**[17]AAGGAAGGCTGGGGGCTGTGTGTGCCTTGGTGACGTGTCCCCCTCGCTCCCGAGCCTT ACTCTGTTCCCCTTTCTTCCTGGCAGCAGAGCCCCCTCCCTTGCTGCACTCAGGGACATGACTGTCAGCACTTCTACCCCCCCTCA GACTTCACTGTCAGCACTCAAGTCTTCAGGGACATGAAAAGGAGCCACTCCTTACAAAAAGTTGGGGAGCCCTGGTGTATTGAG GTAGGTCTCGGAGCAGAATCACGGTATGAGATGAGCTAGCTCTAAAATGAAGCYGCCCACCCAAAGT**TTACCTCTCTCTAGG GCAGGG**[18]CCCATCCACTGYGATCCCTGAAT |{base 139743:C/T}

Exon 8

TATCTATTATTTTCACATGGACTGGGGGGAGTATGGGATGGGCAGAGTTGAGTGCATCTTAGAA**AATCCAGGTGAGAGGCT GTG**[19]GCCAAGGGAACCCACGGGCCCGGTGCTCTGAAGCCCTGGGCTGCTGGAACCGGCTTGACGGTGTCTTTCTCTGTTCTCC TCCCCACAGTCTAACAAAGTGCCAGTGGTGCAGCACCCTCACCATGTCCACCCCCTCACGCMTCTTATCACGTACAG |{base19 1385:C/A}CAATGAACACTTCACGCCGGGAAACCCACCTCCACACTTACCAGCCGACGTAGACCCCAAAACAGGTAGGCTGTGG GCTACGGAGCCAAGGTAGAGTGCTGGTCCTGGGGTTCTGGGGGAACCTTTGAGGCCTCCACAGGAACCCCAGGGG**TGGAGC AGTAGGGGACTTTG**[20]GGGGAACGGTGGTGGGGGGGCCCCTGTTGCTT

Exon 9

CAACAACTCAGGATTTATAATCATGAATGAACCT**TGTGCTAGGATTCCAGAAGAGA**[21]TGCGTCTCTTCCTCCTTTCCCTGTT CCCATCCCCTAATGGATTGCTCTTTCCTTCTCTCCCTCCTTTGTACCTAAAATGCTGCTTCTTCCCTCAGGAATCCCACGGCCTCCG

---

12 TCF4 E9 A1S1 forward

13 TCF4 E9 A2S2 reverse

14 TCF4 E6neu A1S1 forward

15 TCF4 E6neu S2 forward

16 TCF4 E6neu A2S3 reverse

17 TCF4 E15 A1S1 forward

18 TCF4 E15 A2S2 reverse

19 TCF4 E16 A1S1 forward

20 TCF4 E16 A2S2 reverse

21 TCF4 E17neu A1S1 forward

CACCCTCCAGATATATCCCCGTATTACCCACTATCGCCTGGCACCGTAGGACAAATCCCCCATCCGCTAGGATGGTTAGTACCAC
AGTAAGGAGTTCCATTTTTTAATTTCCTTTTTGTTTCTTACATGGGCAYGTTTATTA|{base194222:T/C}TTTATTCTGTGTGTG
TGTSTGTGT|{base194249:C/G}GTGTGTGTGTGTGTGTGTGTGTGTRT|{base194279:G/A}GTRTGTRTGTGTGTGTGTT
ARAAGCCAGGGTTGGGGAGGGGGCTGCGGG AGG**GCACAGAA** |{base194283:G/A}|{base194287:A/G}
|{base194301:G/A}**AAGGTGTT GCTG**[22]AGGTGTCCTGTGGGAATACAGGCAAATCCACAGCCATTCAGGTGGCTT

Exon 10

ACTTTT**CCCTTTTCTTCTTTTTCTTGTC**[23]CCCACCCCCACCCTTGTTTCAAGTCTCTTACTTTGTACCCCTTCTTTGTAGGCAAGG
TCAACCAGTGTACCCAATCACGACAGGAGGATTCAGACACCCCTACCCCACAGCTCTGACCGTCAATGCTTCCATGTCCAGGTGA
GTTCCAAGAACCGGGGCCTTCATCCAAGGCCATGTGTGACTTCTCAAGAAG**TTCTCTAGATGGCCACCAAG**[24]CCACCCAGG
GACCACAGCTACATGTAGTTCTATTAGTGTAAAGCCAATGTGGCATTAGG

Exon 11

TTAATGCTGACAGGTTTCATATGCCT**TGGGTTGCTTTCATGTGAGT**[25]GTTACGTGCTGTTTTTTTTTTTCTTTTTAATTGTGTG
TACACATCCCTCCTCATTCATTCATTTTGATTCTGACGATTTACACAGCTTTCTGTCTTCTAGGTTCCCTCCCCATATGGTCCCACCA
CATCATACGCTACACACGACGGGCATTCCGCATCCGGCCATAGTCACACCAACAGTCAAACAGGAATCGTCCCAGAGTGATGTC
GGCTCACTCCATAGTTCGTAAGTGTTGCTGTTTTTCTCACCTTCTTCGTAGCCGCAGTGTTCTGCAAGCCTGTTGCAGCTGCTGGG
TGGTGGCTTTCTGCCTAAGGTTGGC**CTCGTTTGGTTTGACTGCAG**[26]CCAATACCCAGCCTGTGTGGGCTCTTCACTCCCT

Exon 12

CATTTTCCGGGGTGCAGAAGAACTAAAAGCATGCTTTTTAATCCAAAACTGCTAG**GCTTGGGGGTTATGAGACAA**[27]GGAG
ATACGTTCCCTGCCATGGAGGAAGTTGGACCACGACCTTGTTTATTGGGTTGCGTCTGTTTTGTCTATCTCCAGAAAGCATCAGG
ACTCCAAAAAGGAAGAAGAAAAGAAKAAGCCCCACATAAAGAAACCTCTTAATGCATTCATGTTGTATATGAAGGA |{base20
1910:G/T}AATGAGAGCAAAGGTCGTAGCTGAGTGCACGTTGAAAGAAAGCGCGGCCATCAACCAGATCCTTGGGCGGAGGG
TAGGTGACGCCCTTCTCAGGGAGAAGCKGGGGGCGGGTGGTGAGGG |{base202062:G/T}ACCAGAGTGCAGC
AGGTCAGGTGGCAGAATGTC[28]TCTGTCCCCATTTCTTTGGAGAATTCT

Exon 13

GCAGCATTATTTATCCCCTGA**CCTTGGCGTAATGTGTGATG**[29]TTCTTTCATGCTTTTCTCTTTGTTCATGTCTTTCTCATCTGTA
CCCCACGTCCCCTCCAGTGGCATGCACTGTCCAGAGAAGAGCAAGCGAAATACTACGAGCTGGCCCGGAAGGAGCGACAGCTT
CATATGCAACTGTACCCCGGCTGGTCCGCGCGGGATAACTATGTAGGTGGATCATTTTCGTTAGGATTGGAGTCTGTAGAGCTG
TGTTGTGCGTCTATACGGA**CAAAGAGAACAGGACCTGCC**[30]ACTTGACTAGGGTGGG

Exon 14

GGTTGGAGGTTGGACAAATACCGGGGGTTTGTGTGGATGGAGATGGCAGTATGGTCA**CTTCCTCCTGCCTTCTCCTT**[31]CCAG
GTGCGCCCAGACACTCTTCTCACATCTGTTTCTTGCAGTAATTCCCTGTGTTTCATCTCTTCATCTAGGGAAAGAAGAAGAAGAG

22 TCF4 E17neu A2S2 reverse
23 TCF4 E18 A1S1 forward
24 TCF4 E18 A2S2 reverse
25 TCF4 E19 A1S1 forward
26 TCF4 E19 A2S2 reverse
27 TCF4 E20 A1S1 forward
28 TCF4 E20 A2S2 reverse
29 TCF4 E21 A1S1 forward
30 TCF4 E21 A2S2 reverse
31 TCF4 E23 A1S1 forward

GAAAAGGGACAAGCAGCCGGGAGAGACCAATGGTAAGTGACAATCATCAGGTTAGAGGAAGGAGCTGTAGCCTGAGGACCA
CCCTTTTATGTTAGGTTTCCATCTGGGGGAGG**CAGGAGAAATTCAAGGCCAG**[32]GACATTG

Exon 15

AGGTTTGTCATTCTCTAGAAGAAATTAGAAATACTGCATAGGCAAT**CTCAGAGGTCCCTTGGAGAA**[33]GGCCAGGCTTTTAC
AAACAAACAAAACAAAAATTTTGAAGGCTTTGTATAATTTGTTCTTTTTTTTCAGAACACAGCGAATGTTTCCTAAATCCTTGCCT
TTCACTTCCTCCGATTACAGGTGCTAATGTCATTTTGAGTCATTAAAATAGTTGATAAACTGTTTTTTAATTTTTCTTGCCATTATA
GTTTTATTAACATTTAAA**CACGTATGACATTTGAACATTCTTT**[34]AAAATGACC

Exon 16

AAGTTAAAAAACGAAAGTCAATATTT**TGCAATTAGTAACCAGGTCATTG**[35]ATTTATTCCCTTTTTTATTTTTATTTTTTTMTTA
TTTGTATCTTTCTCTTCCCCCCCCCCCCCCCTCTYTC |{base 209960:C/A} |{base209998:T/C}TCTCTCTCTCTCTCTCCCTT
TCTCCCACGTTCTCCTCCTCTGCTCGCTTCTCTCTTGAACTCATTCAGACCTGAGCGCTCCTAARAAATGCCGAGCGCGCTTTGGC
CTTGATCAACAGAA|{base210085:G/A}TAACTGGTGCGGCCCTTGCAGGTGTGTATAGTTTTCCAGATTCGCTGTGCTGGTT
TGCAGCTGGTCTATTCGATCCTCTCCCCCTTCTCTGGCCTGTTGCTTTGTAGCTCTGTGTGTGGATCTCAG**GAGACACAGGGG
AGTGGGACAC**[36]TGCCAAGTGT

Exon 17

TCTTTTGGCTCATAGAAGTCCTTTCCTTTCA**TGTCCTTGGTGAGGGAAGATT**[37]ATCAAATAGAACCAAGGTGATAGAGAAG
AAAAGGAAGCTGTAGCTGAGATTTCACATCCAACTGAGCACGACCCACCATTGTGTTGTATTTTTTGTGTTTACCTTATGCTAACA
GATGCAAATACTCCAAAGAAGTGTCGGGCACTGTTCGGGCTTGACCGACAGACTTTATGGTGCAAACCGTGCAGGTATATTACC
ACTGCGAGGCCTTTGGGAAAATCAAAGCATTCTGTCCTTCCGGTACCTTAGSGTGATAATT**TATTTTGACCTCGTTCCCCA**[38]T
CTACTTCCCCTCTGGCATCAATGACTAA |{base 210902:C/G}

Exon 18 amplificate 1

CTGGGCGTGCCACCTCTGTGGGACATCCCTTAGGTGACCT**CAGCTTGGGTGTGAGCATTA**[39]GGTAACTCTCTCCCTTGGCAT
CTGTGCCCTCTATTCACAGATAACTCTCTCCCCTGTTTCTAGGAGAAAAAAAAAAGTGCGTTCGCTACATACAAGGTGAAGGCAGC
TGCCTCAGCCCACCCTC**TTCAGATGGAAGCTTACTAGATTCG**[40]CCTCCCCCCTCCCCGAACCTGCTAGGCTCCCCTCCCCGA
GACGCCAAGTCACAGACTGAGCAGACCCAGCCTCTGTCGCTGTCCCTGAAGCCCGACCCCCTGGCCCACCTGTCCATGATGCCT
CCGCCACCCGCCCTCCTGCTCGCTGAGGCCACCCACAAGGCCTCCGCCCTCTGTCCCAACGGGGCCCTGGACCTGCCCCCAGCC
GCTTTGCAGCCTGCCGCCC**CCTCCTCATCAATTGCACAGC**[41]CGTCGACTTCTTCCTTACATTCCCACAGCTCCCTGGCCGGGA
CCCAGCCCCAGCCGCTGTCGCTCGTCACCAAGTCTTTAGAATAGCTTTAGCGTCGTGAACCCCGCTGCTTYGTTTATGGTTTTGTT
TCACTTTTCTTAATTTG |{base216148:T/C}CCCCCCACCCCCACC**TTGAAAGGTTTTGTTTTGTACTCTC**[42]TTAATTTTGT
GCCATGTGGC

32 TCF4 E23 A2S2 reverse
33 TCF4 E24 A1S1 forward
34 TCF4 E24 A2S2 reverse
35 TCF4 E25neu A1S1 forward
36 TCF4 E25neu A2S2 reverse
37 TCF4 E26 A1S1 forward
38 TCF4 E26 A2S2 reverse
39 TCF4 E27 A1S1 forward
40 TCF4 E27 S2 forward
41 TCF4 E27 S3 forward
42 TCF4 E27 A2 reverse

Exon 18 amplificate 2

GCTTTAGCGTCGTGAACCCCGCTGCTTYGTTTAT**GGTTTTGTTTCACTTTTCTTAATTTG**[43] |{base216148:T/C}CCCCCCA
CCCCCACCTTGAAAGGTTTTGTTTTGTACTCTCTTAATTTTGTGCCATGTGGCTACATTAGTTGATGTTTATCGAGTTCATTGGTCA
ATATTTGACCCATTCTTATTTCAATTTCTCCTTTTAAATATGTAGATGA**GAGAAGAACCTCATGATTCTACCAA**[44]AATTTTTA
TCAACAGCTGTTTAAAGTCTTTGTAGCGTTTAAAAAATATATATATATACATAACTGTTATGTAGTTCGGATAGCTTAGTTTTAAA
AGACTGATTAAAAAACAAAAAGAAAAAAAAAGCAATTTTGAAGCAG**CCCTCCAGAAGGAGTTGGTT**[45]CTGTATTATTTGTA
TTAAATACGAGCTTGCGAACCAATCATTTTACATCTGGTTTTTAAACCGTAAGGGCACCATGAATGCAGTGCCGTTACTTTTTTTT
TTTTTTTCTGTGTGAAACAACTCTTATTGTGATGTTACTTGTTATTGTTTAA|{base 216601:-/T/TT} |{base 216602:-
/T/TT} |{base 216606:-/T} |{base 216607:-/T} |{base 216616:-/TT} |{base 216617:-/TT}
ATGTACAGAAACAAAGGGT AAAAATGTGTTAATATA**CCTTGTTCCATGGTGTTGTTC**[46]TTT

Exon 18 amplificate 3

ATGTACAGAAACAAAGGGTAAAAATGTGTTAATATA**CCTTGTTCCATGGTGTTGTTC**[47]TTTTGSGGGGAGGGGACGCTACTC
AACACTTAATAGAATCACAACSCTGTTGGGCCAGTAGTA |{base 216723:G/C} |{base 216763:G/C}
TTTATTGCTTTAGAGATTGCTTGTCGTACCTGTATGTCGTCCCTTTTTAAATATGTTTTCCTTTTTCTTGAAACTGTATAAAGTTTT**T
TTCCCCCTTAGCATAAGCA**[48]TCTTATATATAACAACTCATTTGTACAAGGTTTTTAAGTTTRTATATAAAATGTGTATATATAT
TTTTGTTTC |{base216929:A/G}CCCTTTTTGACTTTTTTTTTTCTGTATGAAACCCAGATGTCACCAAATGGACATTAATAGTT
GCATTAAGGATCAGTAGCATTAACAAAAGTTGCTTTAAAAGCCATTATGTAAAACAAG |{base 217032:-/G} |{base
217037:T/}ACTTGAAAATGAGTGAGGGAATTTTAGCGACACT**GTCTGAGCAGCAGTGGGAAC**[49]CATCTTCGTTTCCCCTTT
GAACTCCCAGTGGGATGCCCTACCCTGYGCCCTTAGGACCCGGACTGA |{base217180:C/T}CCGTGTACAAAACTTTACGTG
CSAAAATTCTCAGTGAATTTAGCTTTCTCCCTCTTTTTG|{base217223:C/G}AT**GCTGTAATTTTTGTTCATCATGTT**[50]TT

Exon 18 amplificate 4

CCGTGTACAAAACTTTACGTGCSAAAATTCTCAGTGAATTTAGCT**TTCTCCCTCTTTTTG** |{base217223:C/G}**ATGCTG**[51]
TAATTTTTGTTCATCATGTTTTGCTGTGATGTTACATAGGTAGATTTGTATGTAGTTTTAATGTCACCTATAACAAAATGTGTTTGG
TAGCAGATTGTCCAGAAAGCATTTTAAATGAAGAGGTATAAACCCTTAAGGGCCAAAATTCTGTATATTAGATTACTCTTAAACG
AAA**AACCAGCTGCCGCTTTTAT**[52]GTACACATATTACATACGAGTAGGCAGCAGACTTTAAAAATAAAAAAAACCTAGGCAT
GTTGATGTTGCAAAATGCTGTATAAAGCTGAAACCTGTTCATTCAGTGCCATTGTAGTTGACATGAAGCGATTGTAAAACTGTCT
CCGATTTTTCTCTGGTTTATTAAAATGCTAACTATAACATTTTTTGTGAATAC**TTTGAATGTTTCCTAACAGTTGTG**[53]ATGTTA
CTGTTCCGTTTTATGCTCTTATTCCRAGTTCATTTTTAATGGTTTGGAAGCCAT |{base217712:A/G}TTTTGTAATGAATAAAT
GTTCATGCTGTACAGTATCTGTAGCATGCCGTTCTGGATTAATAAAAGCAACTTAGTATGTGCARATAAAGGCTGGTCACTTGTT
TCTGTGATTTGGATTTTT |{base217822:G/A}ATCTCTAGGGAAACTGGACCTACCGTAATCAAGAAAAACATATTCCCA**CGT
TGGAACTTGACCATACAG**[54]TTAGTGTGTAGGTGATGACTTAGATACCAGAACGTGGTCTTGGACTCTTT

43 TCF4 E27 A3S4 forward
44 TCF4 E27 S5 forward
45 TCF4 E27 S6 forward
46 TCF4 E27 A4 reverse
47 TCF4 E27 A5S7 forward
48 TCF4 E27 S8 forward
49 TCF4 E27 S9 forward
50 TCF4 E27 A6 reverse
51 TCF4 E27 A7S10 forward
52 TCF4 E27 S11 forward
53 TCF4 E 27 S12 forward
54 TCF4 E27 A8S13 reverse

3. Primer used in Sequenom SNP genotyping assays: all Primers were desalted and had a purification scale level of 0.04 µmol. They were named after the specific SNP which was supposed to be analysed and for which they were designed using Ensemble or SNPdb sequence information and Sequenom software as decribed in the methods part. The ones used in Mulitplex-PCRs for the amplification of sequences harbouring the variations received the additional specification PCR 1 or PCR 2. Primers which where used in the PEX reaction were additionally named Seq. Listed are all primers, even though the assays were later on adjusted in case of SNP exclusion due to compatibility problems or unstable signals.

Assay 1

Oligo	Sequenz
rs11574422 PCR 1	ACGTTGGATGTCTACTCTTCAAACATTCCG
rs11574422 PCR 2	ACGTTGGATGGAACCCAATGGCCATGGAG
rs11574422 Seq	ACATTCCGGCCACTG
rs2417086 PCR 1	ACGTTGGATGGGTCATTTCAGAAAGACTGG
rs2417086 PCR 2	ACGTTGGATGTTTAAACACACTTCACAGG
rs2417086 Seq	CCACTGGCCAGAGAT
rs2277268 PCR 1	ACGTTGGATGGTTGTTATTGGTCTCGAGGG
rs2277268 PCR 2	ACGTTGGATGTGAGTGACATGAAGACCTGC
rs2277268 Seq	TGAAGACCAAGAAGGC
rs2284396 PCR 1	ACGTTGGATGATTTTGGTAGACAGCCTCAG
rs2284396 PCR 2	ACGTTGGATGTACACAGGGAATGAAAACCG
rs2284396 Seq	AGGGAAATTGCTGTGA
rs901824 PCR 1	ACGTTGGATGTAGCACATCCCTGCTCCACT
rs901824 PCR 2	ACGTTGGATGCCCTTCACAGGTAAGGAGC
rs901824 Seq	ttTGCCTCCTCCAGATC
rs2302685 PCR 1	ACGTTGGATGGCCACTTTAGTAACATACCC
rs2302685 PCR 2	ACGTTGGATGAGTTGGAGTGGTGCTGAAAG
rs2302685 Seq	CTCTGGGTTTACCACAA
rs7136380 PCR 1	ACGTTGGATGAGAAAGTAGTCCCGACCACG
rs7136380 PCR 2	ACGTTGGATGAGCGGGATTCTTTCCCGGAC
rs7136380 Seq	CGGCTCCAATGTTGTTC
rs4988322 PCR 1	ACGTTGGATGTGTGGAGGGACTTGGACAAC
rs4988322 PCR 2	ACGTTGGATGTACGAGGTGAACACAAGGAC
rs4988322 Seq	ATCCCACCAAGGGGTAAG
rs41494349 PCR 1	ACGTTGGATGGAGATGACCACGTTCTGCAC
rs41494349 PCR 2	ACGTTGGATGTGTACTGGACAGACGTGAGC
rs41494349 Seq	GTCTGGTTCAGGTAGGTC
rs12416761 PCR 1	ACGTTGGATGAAGCAGAAGTTGAGAGGGAC
rs12416761 PCR 2	ACGTTGGATGAGCGATGGAGGATGTGCGG
rs12416761 Seq	cttcGGCATCCCTGCTGTA
rs35403598 PCR 1	ACGTTGGATGGGATGGAAGCAGACGCTGG
rs35403598 PCR2	ACGTTGGATGAAAGGGGCACGTCAAGGTTC
rs35403598 Seq	tCGGCGCGGTAGAGAGGGC
rs2302686 PCR 1	ACGTTGGATGCAGACCTGGACACCAACTTA
rs2302686 PCR 2	ACGTTGGATGGCCACTGATATTTGCATGGA

rs2302686 Seq	GGACACCAACTTAATAGAAT
rs3736228 PCR 1	ACGTTGGATGAGACTGTCAGGACCGCTCA
rs3736228 PCR 2	ACGTTGGATGTCTTGGCAGAGCCTTGACG
rs3736228 Seq	ggagtACCGCTCAGACGAGG
rs12320259 PCR1	ACGTTGGATGGACAGTTGAAGAAAAAGCTC
rs12320259 PCR2	ACGTTGGATGTTTGGCACATTTCTTGGCAG
rs12320259 Seq	ggacGCTCTTCACCAACCAAC
rs10885395 PCR 1	ACGTTGGATGGAAATGTTAAGTCCCACTAGG
rs10885395 PCR 2	ACGTTGGATGGTTTCTGACATTTAGCATTTG
rs10885395 Seq	ggGTCCCACTAGGATGGATAT
rs34438188 PCR 1	ACGTTGGATGATACCTCTATTCTGAGAGGG
rs34438188 PCR 2	ACGTTGGATGAGCTATATGGCCTGTAGCTG
rs34438188 Seq	ACTAACTGCTTCTCTTTATTTT
rs12424052 PCR 1	ACGTTGGATGGCAATTTCATTCAACAGTAGG
rs12424052 PCR 2	ACGTTGGATGTATAGGAAAAGAGTGTAAG
rs12424052 Seq	TCATTCAACAGTAGGATTACAA
rs4988331 PCR 1	ACGTTGGATGTTTCTCTCAGTGGCAAGAGC
rs4988331 PCR 2	ACGTTGGATGAGAGACAGAGAACAGTGTCC
rs4988331 Seq	tagaCTGATGGGCCAGAGGTTC
rs10885394 PCR 1	ACGTTGGATGAGGAGGAGCAGACTGATTTG
rs10885394 PCR 2	ACGTTGGATGCATTGTGTTTTGGGAGCTGG
rs10885394 Seq	gaGGGGAATGGAATTATGTTAA
rs7308022 PCR 1	ACGTTGGATGGCCCAGCCGATTAACCTTAT
rs7308022 PCR 2	ACGTTGGATGGTCTTTAGATTTTCTCTCTGC
rs7308022 Seq	GAGTGGCTAGTACATGTCATTTA
rs3814570 PCR 1	ACGTTGGATGTGCTGAAACCCCAAACTCTC
rs3814570 PCR 2	ACGTTGGATGAGCACCGAGGTTTCCAAAAG
rs3814570 Seq	ggACCCCAAACTCTCTAAATATTA
rs34143723 PCR 1	ACGTTGGATGCAGTGGGTCATAGTCAATGG
rs34143723 PCR 2	ACGTTGGATGCCGCATGGTGATTGATGAAC
rs34143723 Seq	gaggGGATGGGAAGGATGATGTC
rs682429 PCR 1	ACGTTGGATGAGGAGAAGAATCTCACCCAG
rs682429 PCR 2	ACGTTGGATGAGACTGGGAGTTCCAACTTG
rs682429 Seq	cgaggacGGGCACAGAAGGGGCTC
rs11054706 PCR 1	ACGTTGGATGAGATTACAGGCATGAGCCAC
rs11054706 PCR 2	ACGTTGGATGTAAGACAAATGGAGGTAGAG
rs11054706 Seq	GGTCAAAAAATACGTATTTTAAAAC
rs34743827 PCR 1	ACGTTGGATGATTGCCTCAGGGTTATCTGC
rs34743827 PCR 2	ACGTTGGATGCTCACAACCACAGTAAAGCC
rs34743827 Seq	TTTAATTTTCTCTGTATTTCTTTTTT
rs491347 PCR 1	ACGTTGGATGTGAGCCTGCAGAACTGTATG
rs491347 PCR 2	ACGTTGGATGTTTCATCCTGTCCTGAGAGC
rs491347 Seq	gggtgAACAGGAAGGTTGAGTTAGG
rs7975614 PCR 1	ACGTTGGATGACCATTTGGCCAACCAAGAG
rs7975614 PCR 2	ACGTTGGATGTCCGAAAATTGAGCGAGCAG
rs7975614 Seq	gaagCCAAGAGAAGTGTTAACCAATA
rs11829211 PCR 1	ACGTTGGATGCACAGTTGTTCATGTTATGG
rs11829211 PCR 2	ACGTTGGATGCACATGGATTTGTGGCTGTC
rs11829211 Seq	ccAGTAATCATTGATATTAATCAATGT
rs6488506 PCR 1	ACGTTGGATGGTCAAATAGAGGCTTATATGG

rs6488506 PCR 2	ACGTTGGATGCAAGAGTCATCTTGGTATTCC
rs6488506 Seq	GGCTTATATGGAAAATTTGAAATCATT
rs17848277 PCR 1	ACGTTGGATGACAGCAGATTTGCTGCTTGT
rs17848277 PCR 2	ACGTTGGATGGGTTTAAGGCATGCTTTGTG
rs17848277 Seq	ccccagccGATTTGCTGCTTGTGCCATA
rs11613694 PCR 1	ACGTTGGATGCCAATACATGTACCTAGAGA
rs11613694 PCR 2	ACGTTGGATGAATAGGAGGGATGGATCTCA
rs11613694 Seq	AGATAATAAAATGTAACTTTCAGATATT

Assays 2

Oligoname	**Sequenz**
rs2242339 PCR 1	ACGTTGGATGAACTTGCAGGCCACAGGGTA
rs2242339 PCR 2	ACGTTGGATGACATGATCGAGTCGTCCAAC
rs2242339 Seq	ACGCCCGGCTGCCCC
rs686921 PCR 1	ACGTTGGATGAAACAAGACGGGTTCAATGC
rs686921 PCR 2	ACGTTGGATGAGCGTCATTTATCCAGCACC
rs686921 Seq	TCAATGCCCAAGAGC
rs34216038 PCR 1	ACGTTGGATGAATCTCCCTCTCGCCTGTG
rs34216038 PCR 2	ACGTTGGATGCAAGATCCTGGTGTCGGAG
rs34216038 Seq	GGTGCAGTGCGATGG
rs554734 PCR 1	ACGTTGGATGACAGGTCACAGCTCTCAATG
rs554734 PCR 2	ACGTTGGATGTCTTCACCACCGGCCTCATC
rs554734 Seq	TGCCCAGTGTGTTGTC
rs7302808 PCR 1	ACGTTGGATGCATTCCAGCGGGATTCTTTC
rs7302808 PCR 2	ACGTTGGATGAGAAAGTAGTCCCGACCACG
rs7302808 Seq	aGAGGGCGTGAGGCGG
rs3829909 PCR 1	ACGTTGGATGTTATCAGTGTTGGGACTGGG
rs3829909 PCR 2	ACGTTGGATGAGCCAACTCACACACAACTC
rs3829909 Seq	GTGCCTGGACTGCAACC
rs643981 PCR 1	ACGTTGGATGCTTGGGCTCATGCAAATTCG
rs643981 PCR 2	ACGTTGGATGTCACGGGTAAACCCTGCTG
rs643981 Seq	TGCAAATTCGAGAGAGA
rs11607268 PCR 1	ACGTTGGATGCACGTACAGAACTCCTCCAG
rs11607268 PCR 2	ACGTTGGATGATCGAGCGTGTGGAGAAGAC
rs11607268 Seq	tCAGGCTGACTTCCTCCA
rs10082834 PCR 1	ACGTTGGATGAAGTGACAAGGCTCAATGGG
rs10082834 PCR 2	ACGTTGGATGGTAGAGCTTCTTACCCAACC
rs10082834 Seq	gGAGGACTTAGAGGAACC
rs312778 PCR 1	ACGTTGGATGAACCACACACCCAGCATAAC
rs312778 PCR 2	ACGTTGGATGCCTAATGAGGCCACCTTTAC
rs312778 Seq	ccccAGCACCAGCGGAGAC
rs10219768 PCR 1	ACGTTGGATGGGAAGAAAACGTATTGGTTC
rs10219768 PCR 2	ACGTTGGATGGTGAAGTTTAGATGGTCTCC
rs10219768 Seq	AACTGCGTTTCAAATCGGT
SNP2seq PCR 1	ACGTTGGATGGGAAGCTTACTAGATTCGCC
SNP2seq PCR 2	ACGTTGGATGTCTGCTCAGTCTGTGACTTG
SNP2seq Seq	ttaaTAGATTCGCCTCCCCC
rs2075241 PCR 1	ACGTTGGATGAACACAGTAAGTCCCACAGG

rs2075241 PCR 2	ACGTTGGATGACCCACATGAGTCATTTCTG
rs2075241 Seq	CGGTTAGCACCTTGGAAAAG
rs7294695 PCR 1	ACGTTGGATGGCTTGGAAGAAAGAAAGACC
rs7294695 PCR 2	ACGTTGGATGCCTCTATACCTGTCACCTTC
rs7294695 Seq	ccctAGCCTGATCGATCTGAG
rs17848272 PCR 1	ACGTTGGATGGCATTAAGAGAGTAGTGGGC
rs17848272 PCR 2	ACGTTGGATGAATGTGCTTCCAGCAATGGG
rs17848272 Seq	gggaTGGGCAGGGCATCCACA
rs545382 PCR 1	ACGTTGGATGCAGTCCAGTAGATGAAGTCC
rs545382 PCR 2	ACGTTGGATGATGTTGATGGGACGAAGAGG
rs545382 Seq	cccaCCCCAGCAGCGTGAACCC
rs12424052 PCR 1	ACGTTGGATGGCAATTTCATTCAACAGTAGG
rs12424052 PCR 2	ACGTTGGATGAACGTTCATTTCCTTTCTCC
rs12424052 Seq	TCATTCAACAGTAGGATTACAA
rs12417014 PCR 1	ACGTTGGATGGACAGAGGAGAACATCACAC
rs12417014 PCR 2	ACGTTGGATGTGAGTCCCTCTCAACTTCTG
rs12417014 Seq	cccctCAAAACCCAGGCAATGGC
rs4988321 PCR 1	ACGTTGGATGTCTCCCTCGAGACCAATAAC
rs4988321 PCR 2	ACGTTGGATGCCAGTAGATGTGGTTGTTGG
rs4988321 Seq	ggtTCGAGACCAATAACAACGAC
rs7979485 PCR 1	ACGTTGGATGCTCAAAATATCGTCAGAAGG
rs7979485 PCR 2	ACGTTGGATGCACCAACCACTCTGTTTCAC
rs7979485 Seq	GTCAGAAGGAAAAAATAAGCTAC
rs34618337 PCR 1	ACGTTGGATGTTGACTATGACCCACTGGAC
rs34618337 PCR 2	ACGTTGGATGAGCTCTCAATTACCTGGCTG
rs34618337 Seq	ttggGATCCGAAAGGCACAAGAA
rs34815107 PCR 1	ACGTTGGATGTTATCTGCCAGAGGATGTTG
rs34815107 PCR 2	ACGTTGGATGTAACCAAGAGGCACAGAAGC
rs34815107 Seq	ggggagcGAGGATGTTGTGTCCAC
rs12313200 PCR 1	ACGTTGGATGTCACTTCTGATCATAGTGCC
rs12313200 PCR 2	ACGTTGGATGCATTTCTCAATTTTCAAAGC
rs12313200 Seq	TTCCATATTATTGTTTTAATTCACT
SNP1seq PCR 1	ACGTTGGATGTACATACGAGTAGGCAGCAG
SNP1seq 1stPCRP	ACGTTGGATGGCACTGAATGAACAGGTTTC
SNP1seq Seq	CAGCAGACTTTAAAAATAAAAAAAA
rs4638401 PCR 1	ACGTTGGATGTTCAGGGCGTGGTATGTATG
rs4638401 PCR 2	ACGTTGGATGGAGGGCTTGCTAATAGGATG
rs4638401 Seq	GTGGTATGTATGTACTCATAAAAAA

Assay 3

Oligoname	**Sequenz**
rs1012672 PCR 2	ACGTTGGATGAACTGGGACTCTGAGCATAC
rs1012672 PCR 1	ACGTTGGATGATTGACTGTATCCCTGTGGC
rs1012672 Seq	TGTGGCTTGGCGGTG
rs2417085 PCR 2	ACGTTGGATGGAAGAATATCTGTAGCAAG
rs2417085 PCR 1	ACGTTGGATGCTAAGATGGGCAAAACCAAA
rs2417085 Seq	TTGGTGCATTCCCTTA
rs7304561 PCR 2	ACGTTGGATGCAATCACTTGAACCTGGGAG

rs7304561 PCR 1	ACGTTGGATGGAGACGGAGTTGTGCTCTTG
rs7304561 Seq	ccGCTCTTGCTGCCTGG
rs12314349 PCR 2	ACGTTGGATGCCACGTGTGAGATCATGTAG
rs12314349 PCR 1	ACGTTGGATGTGCAACAACACAGATGAACC
rs12314349 Seq	ACAGATGAACCTGGAAG
rs10845494 PCR 2	ACGTTGGATGTAATCCATGCATCTATAGCC
rs10845494 PCR 1	ACGTTGGATGGAAAAGACTATCCTTTCCCC
rs10845494 Seq	ATCCTTTCCCCAATCTAT
rs34426182 PCR 2	ACGTTGGATGTGCCCAGCTTAGTGACATTC
rs34426182 PCR 1	ACGTTGGATGCCTCCAATTAGCTTTATCCC
rs34426182 Seq	TTCTTGAAGGTTCAGCTC
rs11609634 PCR 2	ACGTTGGATGGGAAAAGCGGGAGAGAATAG
rs11609634 PCR 1	ACGTTGGATGCCTCCCCTTACTCATTCAAC
rs11609634 Seq	ACAACCTAATCTCTGTCTT
rs10743980 PCR 2	ACGTTGGATGGGTGGATTTCTTTCTGCAGG
rs10743980 PCR 1	ACGTTGGATGGATAAACACCCTTATCCGAAG
rs10743980 Seq	CCTTATCCGAAGTGAAAACA
rs10772542 PCR 2	ACGTTGGATGCTCTCTCTAAATTTGGCCTC
rs10772542 PCR 1	ACGTTGGATGTCTTCCCGTTGCTTAGTCTG
rs10772542 Seq	GTTGCTTAGTCTGAACTGTA
rs11054701 PCR 2	ACGTTGGATGCTTAATATAGGAGAATTCTT
rs11054701 PCR 1	ACGTTGGATGGACTCCATCTCTACAAAAAAC
rs11054701 Seq	ACAAAAAATTGCTAGGGAGAA
rs7978064 PCR 2	ACGTTGGATGCCATTCTCCAGGAGTTTGAC
rs7978064 PCR 1	ACGTTGGATGTTGATGTCTCCAGTCAAGCC
rs7978064 Seq	CAGTCAAGCCTTTTTATCAGTG
rs17848270 PCR 2	ACGTTGGATGTGGGTTGATCCAACTCTTGC
rs17848270 PCR 1	ACGTTGGATGCAGATTCTGAAACTAATCGG
rs17848270 Seq	TGAAGTTTCTAATTTAGATGGA
rs1181334 PCR 2	ACGTTGGATGATTCCCCCAACCAGGAAAAC
rs1181334 PCR 1	ACGTTGGATGCTAAGTCATGTGAGAACTTTC
rs1181334 Seq	ATGTGAGAACTTTCTTTTTCCTT
rs12309338 PCR 2	ACGTTGGATGCTTTCCCCAGGGTAGTTTTG
rs12309338 PCR 1	ACGTTGGATGCCTCAGTGACATTGGAGTTC
rs12309338 Seq	cccccTGCTTGAACACAAGTTATC
rs1420734 PCR 2	ACGTTGGATGTAGTCTTGTCCAATTGTCCC
rs1420734 PCR 1	ACGTTGGATGTTAAGCCCGACCAGCTAAAG
rs1420734 Seq	agtaCAAGATAAATGCCTCAAAAC
rs7975614 PCR 1	ACGTTGGATGTCCGAAAATTGAGCGAGCAG
rs7975614 PCR 2	ACGTTGGATGACCATTTGGCCAACCAAGAG
rs7975614 Seq	AACCAAGAGAAGTGTTAACCAATA