From THE DEPARTMENT OF MICROBIOLOGY, TUMOR
AND CELL BIOLOGY (MTC)
Karolinska Institutet, Stockholm, Sweden

Biochemical parameters reflecting the intestinal ecology of healthy adults and alterations of these parameters in patients with ulcerative colitis or rheumatoid arthritis

Peter Benno

Karolinska
Institutet

Stockholm 2009

ABSTRACT

The gastrointestinal microflora, the largest single collection of cells in the body, influence their human host in a variety of ways. Useful in this connection is the concept of Microflora-Associated Characteristics (MACs), which are defined as any anatomical structure or physiological, biochemical or immunological function in the host that is affected by the microflora.

In the present investigation we have established and evaluated reliable reference values for three MACs, i.e., the conversion of cholesterol to coprostanol, content of urobilinogens and tryptic activity (FTA) in faecal samples. Ours is the largest study of its kind to date, assessing these parameters in more than 560 healthy men and women (HS; Healthy Subjects) and analyzing the findings with respect to both gender and age (employing arbitrary division into groups < 36, 36-50 and > 50 years old).

Conversion of cholesterol to coprostanol in 633 HS; In general, the proportion of men with the highest conversion rate was found in the oldest age group in contrast to women were the conversion was of the same order of magnitude, irrespectively of age group.

One possible explanation for these observations might be that men with low or no converting capacity in the oldest age group are lost in this cohort of HS. However, to rule out whether the capacity to convert cholesterol is related to morbidity requires additional long-term studies.

Formation of urobilinogens in 562 HS; In general, the levels of urobilinogens were higher in men irrespectively of age group.

Both bilirubin and its metabolites – urobilinogens – undergo enterohepatic circulation and thereby provide an interesting endogenous model for xenobiotics. The reference values found in this study might be of value when evaluating metabolism of xenobiotics in relation to gender and age.

Degradation of FTA in 573 HS; In general, the mean levels were highest in the youngest male group and decreased successively in the two older groups. Although the same tendency was observed among the women, at all ages the men demonstrated higher mean values.

FTA appears to be an important regulator of the intestinal flora by activating defensins. The clinical relevance of these findings in patients with inflammatory bowel disease should be explored in greater details.

Determination of these same MACs in patients suffering from Ulcerative Colitis (UC) or Rheumatoid Arthritis (RA): Irrespective of the extent to which their disease had spread, patients suffering from UC exhibited less formation of coprostanol, reduced levels of urobilinogens and elevated levels of FTA in comparison to HS. For ethical reasons we could not clarify the influence of SalicylAzoSulphaPyridine (SASP; sulphasalazine) on these variables. Thus, patients with RA treated with SASP were evaluated with respect to these MACs as well.

Patients with RA who were not receiving SASP demonstrated a higher frequency of high converters of cholesterol to coprostanol in comparison to a gender- and age-matched group of HS. Treatment with SASP in patients with RA tended to reduce the coprostanol formation but markedly reduced the levels of urobilinogens and increased the FTA values compared to the untreated patients with RA.

Consequently, it appears likely that the alterations observed in patients with UC are also induced by SASP, which seems thus to exert a general influence on the cross-talk between different types of microbes and between microbes and the host which goes on continuously in the gastrointestinal tract.

Keywords: Microflora-associated characteristics; cholesterol; coprostanol; urobilinogen; faecal tryptic activity; ulcerative colitis; rheumatoid arthritis; sulphasalazine

LIST OF PUBLICATIONS

The present thesis is based on the following publications, which will be referred to in text by their Roman numerals:

I   Benno P, Midtvedt K, Alam M, Collinder E, Norin E & Midtvedt T.
Examination of intestinal conversion of cholesterol to coprostanol in 633 healthy subjects reveals an age- and sex-dependent pattern.
Microb Ecol Health Dis 2005;17:200-204.

II   Benno P, Alam M, Collinder E, Norin E & Midtvedt T.
Faecal tryptic activity and excretion of urobilins in 573 healthy subjects living in Sweden, Norway and Scotland.
Microb Ecol Health Dis 2003;15:169-174.

III   Benno P, Leijonmarck C-E, Monsén U, Uribe A & Midtvedt T.
Functional alterations of the microflora in patients with ulcerative colitis.
Scand J Gastroenterol 1993;28:839-844.

IV   Benno P, Alam M, Henriksson K, Norin E, Uribe A & Midtvedt T.
Abnormal colonic microbial function in patients with rheumatoid arthritis.
Scand J Rheumatol 1994;23:311-315.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

FTA	Faecal Tryptic Activity
GAC	GermFree Animal Characteristic
GF	GermFree
GI	GastroIntestinal
IBD	Inflammatory Bowel Disease
MAC	Microflora-Associated Characteristic
ME	Milieu Exterieur
MI	Milieu Interieur
MT	Milieu Total
NSAID	Non-Steroidal Anti-Inflammatory Drug
PPAR-γ	Peroxisome Proliferator-Activated Receptor-γ
PSTI	Pancreatic Secretory Trypsin Inhibitor
RA	Rheumatoid Arthritis
SASP	SalicylAzoSulphaPyridine (sulphasalazine)
SCFAs	Short-Chain Fatty Acids
SP	SulphaPyridine
UC	Ulcerative Colitis
5-ASA	5-AminoSalicylic Acid

1 INTRODUCTION

The GastroIntestinal (GI) flora is the most complex part of the mammalian microflora, with a composition that differs between animal species and between different members of the same species, as well as during the lifetime of one and the same individual. The extensive efforts devoted to mapping the composition of the intestinal microflora have resulted in the isolation, characterization and enumeration of hundreds of bacterial species. Such detailed analysis, involving molecular approaches, is a monumental task. An alternative strategy has been to examine the metabolic capacity of the microbial flora *in vitro* [Midtvedt 1974].

A third approach is to investigate the overall effect of interactions between the microflora and their host, - i.e., the functional homeostasis established by the host, the intestinal flora and the intraluminal environment in combination (Figure 1).

Macroorganism
species
age
sex
diseases

Intestinal microflora
composition
function

Environment
diet
drugs
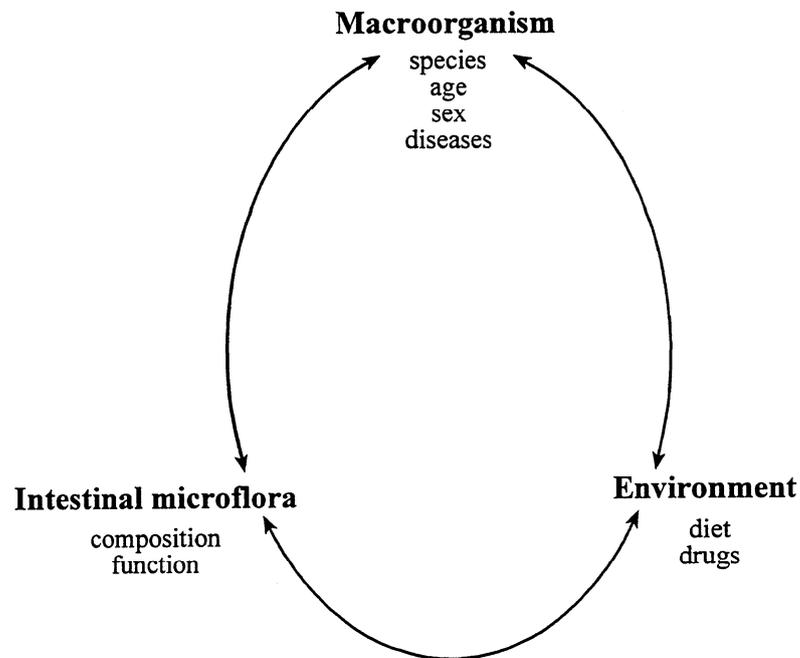
Figure 1. The gastrointestinal ecosystem in man and animals.

Elucidation of these interactions, referred to as Microflora-Associated Characteristics (MACs), has proven to be useful in evaluating the GI ecosystem [Midtvedt 1985a]. MACs are defined as the recording of any anatomical structure, physiological, biochemical or immunological function in a macroorganism, that is influenced by the microflora.

Over the years, numerous attempts have been made to assess the role played by the microflora in the pathogenesis of Inflammatory Bowel Disease (IBD) and Rheumatoid Arthritis (RA). To date, however, the gut flora of patients with these diseases have not been found to exhibit any distinguishing characteristics and, furthermore, no consistent changes in the composition of

their faecal flora following treatment with sulphasalazine (SalicylAzoSulpha-Pyridine; SASP), a drug commonly used in their treatment, have been reported.

2 BACKGROUND

2.1 The gastrointestinal microbiota: general aspects

The qualitative and quantitative composition of the microflora in the various compartments of the GI tract reflect complex interactions between the microorganisms themselves, as well as with the host. These interactions can be characterized on several different levels (e.g., phenotypic and genotypic alterations, etc.) and the outcome is often described in terms such as commensalism, parasitism, mutualism, antagonism, etc. It should be emphasized, however, that such terms most often refer to the outcome for the host, not for the individual microbial species or strain.

The GI tract harbours bacteria that display pronounced structural differences. In Gram-negative bacteria, the cell wall consists of lipopolysaccharide layer, outer membrane, lipoprotein and peptidoglycan layer. The lipopolysaccharide layer contains a complex lipid, lipid A, which is released when the cells are lysed and is extremely toxic both to human beings and other mammals [Jawetz et al. 1987]. Furthermore, lipopolysaccharides activate innate immunity, stimulating lymphocytes and the release of cytokines [Mattern et al. 1994], thus promoting the development of inflammatory responses. The cell wall of Gram-positive bacteria consists mainly of peptidoglycan and teichoic acids. These peptidoglycans can trigger the immune system and induce severe experimental arthritis in rats (Hazenberg et al. 1992).

The distribution of microflora in different compartments of the intestine is influenced to a considerable extent by the host, as well as by environmental factors. In the case of man, only a few species, such as *Helicobacter pylori,* are capable of colonizing the highly acidic gastric environment (pH 1-2). As the contents of the small intestine become more alkaline, the numbers and complexity of the resident flora gradually increase. Several factors, including the

i. rapid transit of contents due to pronounced motility [Huseby et al. 2001],
ii. influence of clearance by bile acids [Floch et al. 1972],
iii. local actions of IgA [Neutra & Forstner 1987] and
iiii. defensins [Wehkamp & Stange 2006]

counteract the establishment of resident microbiota in the upper portion of the small intestine. In contrast, in the lower regions of the small intestine and in the colon, the population of bacteria, and in particular of anaerobes, increases markedly, being as much as a million-fold higher in the colon than in the upper small intestine.

2.1.1   Interactions between the host, intestinal flora and environment

Interactions between intestinal bacteria and the host may vary from being primarily parasitism to being largely mutualism. In parasitism one of the partners benefits at the expense of the other, whereas mutual relationships are beneficial for both. In connection with both of these types of interactions, the gut microbes carry out a large number of biochemical reactions and can actually be considered to be the most active and largest "organ" in the human body.

As might be expected, the nature of the diet extents major influences on both the composition and metabolic activities of the intestinal microflora. In terms of bulk, carbohydrates constitute the largest portion of human diets and disacharides such as sucrose, lactose and maltose are hydrolyzed to their constituent monosacharides in the lumen of the host intestine, where starch is also broken down to glucose [Hooper et al. 2002]. In the small intestine hydrolysis of other polysaccharides occurs to only a limited extend, as a result of which undegraded carbohydrates represent a significant proportion of the dietary components that pass into the colon each day.

Fermentation of these carbohydrates by bacteria under the anaerobic conditions present in the lower regions of the gastrointestinal tract results in organic acids, predominantly Short Chain Fatty Acids (SCFAs). SCFAs longer than acetic acid, including propionic, butyric, caproic and valeric acids, are produced by anabolic bacterial processes that consume electrons and ATP. Oxidation of butyric acid, an important fuel for the host enterocytes, is required for the absorption of ions, and synthesis of mucin and lipid components of colonocyte membranes [Roediger 1980]. This is a good example of multistep interactions between the microflora and their host.

The presence of large quantites of animal protein in the diet elevates the activities of certain bacterial enzymes, including β-glucuronidase, in both animals and humans [Hawrelak & Myers 2004], a phenomen which can have important ramifications for the host. For instance, many xenobiotics are conjugated, with, e.g., glucoronic acid to enhance their solubility in water prior to elimination from the body [Mital & Garg 1995]. Removal of the glucuronic acid moiety from such conjugates excreted in the bile by the β-glucuronidase of the intestinal microflora reduces their hydrophilocity and allows them to reenter the body. A molecule may undergo such enterohepatic circulation several times before eliminated in the faeces [Saxerholt 1990]. If the original compound and/or its metabolites are toxic and/or genotoxic this may enhance the risk for deleterious effects by prolonging its half-life in the body.

Moreover, the important role that microbes play in helping to extract maximal nutrition from the host´s diet is demonstrated clearly by the observation that conventional animals require lower caloric intake in order to maintain their body weight in comparision to their Germ-Free (GF) counterparts [Wostmann 1983].

4

An additional important aspect of the dualistic relationship between the flora and their host concerns the metabolism of some nitrogen compounds. When formed in the liver, major nitrogen-containing products of protein degradation in all mammalian species, will be either excreted into the urine or diffuse into certain compartments of the GI tract.

Urease, an enzyme expressed by many GI microbes, hydrolyse urea to yield ammonium, which is utilized by the microbes for their own growth and production of enzymes for hydrolysis of carbohydrates to produce SCFA, which provide energy to the host [Midtvedt 2006]. Furthermore, ingested nitrate, another nitrogen-containing compound, is taken up from the GI tract and within minutes some of this is subsequently secreted into the saliva. In the mouth, bacterial reductases reduce nitrate to nitrite. In the acid stomach environment nitrite is non-enzymatically reduced further to nitric oxide. Both nitrit and nitric oxide may influence upon many bacterial species as well as the host itself [Sobko 2006].

In addition, intestinal flora represents an important source of vitamins for the host. Thus, GF rodents require vitamin K in their diet, in contrast to animals raised in a conventional manner [Gustafsson 1959]. Some GF animals also require supplementation with certain of the B vitamins that are synthesized by intestinal microbes.

2.2   Approaches to the evaluation of intestinal bacteria

2.2.1   Culturing

Isolation and identification of bacterial species from the GI tract requires specialized techniques, e.g., the use of various selective media in combination with anaerobic culture conditions [for a review, see Washington 1991]. In attempt to mimic the complex ecosystem present, *bioreactors* designed to simulate the environmental conditions in the gut have been constructed [Mäkivuokko & Nurminen 2006]. Despite the advanced techniques employed, such models obviously lack the numerous interactions that occur between the host and flora.

2.2.2   Morphological, molecular and biochemical approaches

Microscopic examination, usually after various types of staining, allows the detection and partial identification of microorganisms and reveals the general features of their morphology [Washington 1991].

Molecular methods are the most precise method for classifying bacteria by analysing their genetic material. DNA hybridisation was used initially to determine the relationship among bacterial isolates. By using species-specific probes it is also possible to identify bacteria in clinical material [Norin et al. 2009].

Biochemical tests based on the ability to metabolize various compounds primarily carbohydrates, provide valuable characterization of bacteria [Miller & O´Hara 1995]. Alterations in the *in vitro* metabolic capacity of the microflora isolated from patients with UC and their relatives have been demonstrated in this manner [Katouli et al. 1992].

Evaluation of Microflora-Associated Characteristics (MACs). Applying a slight travesty of the well-known terminology introduced by Claude Bernhard, the mammalian organism can be considered to be a Milieu Interieur (MI), a normal microbiota as a Milieu Exterieur (ME) and the combination of these two a Milieu Total (MT) [Midtvedt 1989]. In evaluating the interplay between MI and ME, two concepts, i.e., Microflora Associated Characteristic (MAC) and Germfree Animal Characteristic (GAC), have been proven to be of considerable value. As mentioned previously, a MAC can be defined as any anatomical structure or physiological, biochemical or immunological function of a macroorganism which is influenced by the microflora. GF animals represent the MI alone and thus, through comparision with their normal/conventional counterparts, provide valuable indirect information concerning MACs involving GI parameters [Midtvedt 1985a, Midtvedt et al. 1985b, Midtvedt 1999]. When we are investigating conventional organisms – MT – the question "what have the microbes done, "can be answered by the equation MT minus MI = ME. Table 1 lists some of the many anatomical, physiological and biochemical parameters which have been investigated employing GF organisms and three of the MACs studied most commonly are described below and have played a central role in the work described here.

Conversion of cholesterol to coprostanol. Cholesterol is both an important component of the cellular membranes of all mammals and the precursor for the biosynthesis of steroid hormones, vitamin D, and bile acids. Pathophysiologically, this compound is also thought to be involved in the pathogenesis of atheromatous arterial disease, hypertension and cancer of the large bowel.

The cholesterol present in the intestinal contents originates from both endogenous and exogenous sources. Endogenous cholesterol is mainly produced by synthesis in the liver and small intestine while exogenous cholesterol is derived from food of animal origin. The major routes for the elimination of plasma cholesterol involve hepatic conversion of this molecule to bile acids and subsequent biliary excretion of both these bile acids and unmetabolized cholesterol.

In the intestinal tract, this biliary cholesterol is mixed with endogenous cholesterol produced by the mucosal cells, as well as with dietary cholesterol, and can be (re)absorbed or metabolized by the intestinal microflora.

Coprostanol, the major bacterial metabolite in both human beings and other mammals is eliminated entirely in the faeces, where it constitutes the major derivative of cholesterol [Benno 1999]. Until recently, formation of

6

coprostanol was thought to be performed by only a few species of bacteria, e.g., *Eubacterium lentum* and *E. coprostanoligenes*, which are strictly anaerobic, Gram-positive rods [Eyssen et al. 1973, Sadzikowski et al. 1977, Freier et al. 1994, Ren et al. 1996]. However, a *Bacteroides* strain was recently isolated from human faeces and was shown to be capable of catalyzing this transformation [Gérard et al. 2007].

Conversion of bilirubin to urobilinogens. Bile pigments, which consist mainly of conjugated forms of bilirubin, are the end-products of the catabolism of the heme moiety of hemoglobin and the other hemoproteins in the body. The highly hydrophobic bilirubin molecules are conjugated with glucuronic acid in order to enhance their water-solubility and thereby facilitate excretion in the bile. In the intestine, conjugates are deconjugated and transformed into a series of urobilinogens [for a review, see Saxerholt 1990]. A minor portion of intestinal β-glucuronidase activity may be expressed by the host, but most is of microbial origin [Rød & Midtvedt 1977]. The ability to transform bilirubin into urobilinogens seems to be a rare event among intestinal microorganisms [Midtvedt & Gustafsson 1981, Vítek et al. 2005].

Conjugation of bilirubin - and of several xenobiotics - can be regarded as a general detoxificating pathway [Saxerholt 1990] for excreting potentially toxic endogenous and exogenous products. Studies involving children and adults, as well as rats, mice, pigs and horses demonstrated that any organism with a normal microbiota can carry out this conversion [for a review, see Collinder 2001].

Inactivation of tryptic activity. In respons to the intake of food containing proteins, inactive trypsinogen is secreted by the pancreatic gland together with PSTI (<u>P</u>ancreatic <u>S</u>ecretory <u>T</u>rypsin <u>I</u>nactivator), into the small intestine where trypsinogen is activated to trypsin either by enterokinase or active trypsin already present. Until recently, PSTI was thought to be present only in the pancreas gland, but has subsequently been identified in mucin producing cells throughout the GI tract [Marchbank et al. 1998]. FTA is the net result of complex interactions that occur between pancreatic trypsinogen and activators and inactivators that are produced by the host, or intestinal flora or are present in the diet [Norin 1989]. In general, FTA is attenuated by intraluminal bacteria, as revealed by the high level of this activity present in GF rats [Norin et al. 1986a, Norin et al. 1986b]. This inactivation of trypsin occurs primarily in the caecum [Norin et al. 1986b] and has been shown to be carried out by e.g., *Bacteroides distasonis* [Ramare et al. 1996].

Table I. Influence of the microflora on certain major anatomical, physiological and biochemical parameters of the intestine.

Parameter	MAC*	GAC*	Microbes
Anatomical/physiological			
Intestinal wall	Thick	Thin	Unknown
Cell kinetics	Fast	Slow	Unknown
Migration motor complexes	Normal	Fewer	Unknown
Size of the caecum	Normal	Enlarged	Partly unknown
Oxygen tension	Low	As high as in tissues	Several species
Redox potential (mV)	Low (< -100)	High (>0)	Unknown
Biochemical			
Bile acid metabolism	Deconjugation	No deconjugation	Several species
Dehydrogenation	Dehydrogenation	No dehydrogenation	Many species
	Dehydroxylation	No dehydroxylation	Few species
Bilirubin metabolism	Deconjugation	Little deconjugation	Many species
	Formation of urobilinogens	No formation of urobilinogens	Few species
Cholesterol	Formation of coprostanol	No formation of coprostanol	Few species
Intestinal formed gases	Carbon dioxide	Some carbon dioxide	Many species
	Hydrogen	No hydrogen	Some species
	Methane	No methane	Few species
Mucin	Degradation	No degradation	Many species
Tryptic activity	Little or none	High activity	Few species
β-aspartylglycine	None	Present	Little known
Formation of SCFAs	Large amounts	Far less**	Many species

these Microflora Associated Characteristic (MAC) and Germfree Animal Characteristic (GAC), are

* adapted from T Midtvedt (1999).

** mainly acetic acid from the diet

2.3 *The intestinal flora and disease*

There are certain areas of similarity between gastroenterology and rheumatology which probably reflects common, still partly unknown pathogenic mechanisms and also possible influence(s) on these mechanisms of commonly used drugs in both IBD and RA. The intestinal flora is believed to be one factor which may play a key role in trigger inflammation in the bowel such as in UC, as well as in connection with extraintestinal systemic disorders like RA. Undesirable alterations in the gut flora has been referred to as "dysbiosis" by Metchnikoff and simply as "qualitative and quantitative change in the intestinal flora, their metabolic activity and their local distribution" by others [Holzapfel et al. 1998].

2.3.1 Ulcerative Colitis (UC)

Background. Ulcerative proctocolitis refers to a chronic inflammation of the mucosa characterized clinically by asymptomatic periods. The incidence of this disease in Sweden varied between 4-13 new cases per 100,000 inhabitants each year [Nordenvall et al. 1985, Tysk & Järnerot 1992] and its prevalence was estimated to be approximately 230 cases per 100,000 people. Onset usually occurs between the ages of 20 and 40 [Tysk & Järnerot 1992].

The inflammation can be restricted to the rectum (proctitis), or the left flexure (left-sided colitis) or can afflict the entire mucosa of the large bowel (total colitis). Rectosigmoidoscopy/colonoscopy and examination of biopsies provide valuable information in connection with clinical attempts to diagnose UC. Common histological characteristics include infiltration by inflammatory cells, basal plasmocytosis, abnormal architecture of the crypts, and a villous-like mucosa, along with mucosal atrophy [Schumacher 1993].

The extraintestinal manifestations of UC include arthralgias and, in 20% of the cases, arthritis. The involvement of major joints is usually asymmetric, but when symmetric, this condition resembles RA. Joint inflammation may persist for weeks to months, but is usually reversible, and persistent deformity of the joints involved is rare [Weiner et al. 1991].

Etiopathogenic aspects. In connection with UC tissue damage occurs in those regions of the mucosa that are heavily infiltrated with active CD4+ T lymphocytes. Markedly elevated levels of inflammatory mediators, including eicosanoids, leukotrienes, free radicals, homocysteine, pro-inflammatory cytokines and chemokines, are found in these areas [Stokkers & Hommes 2004, Danese et al. 2005, Puleston et al. 2005].

In recent years the participation of mast cells in the pathogenesis of UC has been demonstrated [Raithel et al. 2001]. Among other proinflammtory mediators activated mast cells release tryptase, one of the serine proteases that exhibit trypsin-like activity. Employing an experimental model of IBD, Cenac and coworkers [2002] found that intraluminal administration of tryptase induces inflammation of the colon. Also of interest in this context is the observation that intrarectal administration of nafamostat mesilate, a protease inhibitor, commonly used in the treatment of acute pancreatitis, reduces both inflammation in UC patients and trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats [Yoshida et al. 2006].

In a number of experimental animals that have been genetically engineered in order to produce different systemic immune disorders, intestinal inflammation also occurs. This is the case for mice deficient in the production of IL-2, IL-10 or TCRα, but, interestingly, when these same animals are made GF, they do not develop inflammatory bowel disease [Elson et al. 1998]. These observations demonstrate that the intestinal flora is necessary and promotes the inflammatory process, at least in these animal models.

Although clinical data indicate that UC might occurs in connection with transient intestinal infections (like traveller´s diarrhoea), reliable evaluation of the alterations in the microflora associated with this disease is lacking. Several studies have revealed no consistent changes in the intestinal flora in patients with UC [van der Wiel-Korstanje & Winkler 1975, Keighley et al. 1978, Dickinson et al. 1980, Hartley et al. 1992, Bullock et al. 2004].

Nonetheless, treatment with probiotic agents has been attracting greater interest during recent years. Some pilot studies and clinical trials have evaluated the efficacy of a probiotic preparation known as *E.coli Nissle 1917*. Interestingly, this non-pathogenic bacteria proved to exert effects on patients with UC that were as beneficial as those obtained using standard maintenance treatment with mesalazine (5-AminoSalicylicAcid; 5-ASA) [Rembacken et al. 1999, Kruis et al. 2004].

The mechanisms that have been proposed to underlie the general effects of probiotics include counteracting the adherence [Johnson-Henry et al. 2007] and/or translocation [Osman et al. 2006] of pathogenic bacteria or the production of bacteriocins [Stern et al. 2006]. Furthermore, probiotics may stimulate mucosal defences and help to achieve a balanced T-helper cell response [Forchielli & Walker 2005]. Whether and to what extent these proposed effects have any bearing in patients with UC remains to be proven.

A small number of limited studies have addressed the possible beneficial effects of prebiotics on UC. For instance, Fernández–Banares and coworkers [1999] found that treatment of patients with UC with psyllium (also known as ispagula or Plantago ovata) was as effective as conventional maintenance treatment with mesalazine.

Taken together, the prophylactic and therapeutic potential of pre- and probiotics in connection with UC is still far from elucidated.

2.3.2   Rheumatoid Arthritis (RA)

Background. The primary symptom of RA, a chronic inflammatory systemic disease, is arthritis in the peripheral joints, usually exhibiting a symmetrical distribution. Diagnosis of RA is based on various clinical features, radiographic changes, presence of anticyclic citrullinated peptides [Samanci et al. 2005] and IgM autoantibodies against IgG (i.e., rheumatoid factor) in the serum [Arnett et al. 1988]. The functional status of these patients is assessed with a number of scales developed specifically for this purpose, of which one of the most commonly empoyed is the Stanford Health Assessment Questionnaire (HAQ) [Ekdahl et al. 1988]. This questionnaire provides a quantitative evaluation of functional status, including the limitations of self-care.

The prevalence and estimated incidence of RA are approximately 1% and 0.01%, respectively [Gran 1987], with this disease being 2- to 3-fold more common in women than in men [Alamanos et al. 2006]. Onset, which may occur at any age, is most common during the fourth and fifth decades of life.

As mentioned above, the hallmark of RA is bilateral, symmetrical polyarthritis, most characteristically involving both wrists and the second and third metacarpal phalangeal joints. Its severity varies from mild, where only a few joints are slightly affected, to severe, with irreversible tissue damage associated with deformation and instability of joints. A wide variety of other manifestations common to rheumatoid disease are also found in patients with RA, such as rheumatic nodules, vasculitis, and complications involving the heart, lungs, kidneys and skin [Zvaifler 2006].

At an early stage in the development of the disease inflammatory cells infiltrate the synovial membrane, followed by proliferation of mononuclear cells and resident synovial cells, which leads to hypertrophy of the synovial membrane and joint effusions.

Etiopathogenic aspects. On the basis of their presence in elevated numbers in the gut and throat of patients with RA, Svartz [1972] suggested that *Streptococcus sp* was a trigging factor for RA. Other abnormalities in the bacterial flora of these patients, in particular with regards to *Clostridium perfringens* [Olhagen & Månsson 1968, Shinebaum et al. 1987] and *Proteus mirabilis* [Ebringer et al. 1989, Rogers et al. 1988], have also been detected utilizing culturing and serological techniques [Sapico et al. 1973, McDonagh et al. 1994]. Moreover, in biopsies of the jejunal mucosa and samples of intestinal fluid, Henriksson and coworkers [1993] found a frequent intestinal bacterial overgrowth, in association with the active disease. Additional observations suggest that the intestinal microflora modulate the development of arthritis. For example, Kohashi and colleagues [1985] demonstrated that injection of bacterial cell walls into rats can induce arthritis and, furthermore, colonization of GF rats with a single species of Gram-positive bacteria aggravates arthritis, whereas Gram-negative bacteria exert the opposite effect.

Employing an experimental model referred to as "adjuvant arthritis", we have shown that when conventional male and female rats are injected with whole *M. tuberculosis* cells suspended in Freund´s incomplete adjuvant, the females develop arthritis that is significantly more severe. Moreover, the susceptibility of the male animals is more age-dependent, with younger rats demonstrating more severe disease. These observations illustrate clearly that host factors such as age and sex can influence inflammatory responses to an exogenous agent [Benno et al. 1996a].

Interestingly, in a more comprehensive investigation involving three experimental models of arthritis in both GF and conventional rats of an arthritis sensitive strain (DA), the incidence of severe arthritis observed in all three models was independent of microbial status. No antibodies to heat-shock protein 65 could be detected in the serum of the GF animals, in contrast to the conventional animals [Björk et al. 1994].

In many patients with RA the GI mucosa appears to be affected. For example, chronic inflammation and vasculitic lesions have been observed in gastric, colonic and rectal biopsies from such patients [Marcolongo et al. 1979] and an enhanced incidence of villous atrophy has also been reported

[O´Farrelly et al. 1988]. Moreover, alterations in the intestinal permeability in association with administration of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) [Bjarnason et al. 1993], and an elevated frequency of atrophic gastritis resulting in achlorhydria and bacterial overgrowth have been observed [Henriksson et al. 1986, Di Mario et al. 1989]. However, no consistent relationship between such changes and abnormal interactions between the microflora and intestinal mucosal cells has yet been definitively demonstrated.

2.4 Sulphasalazine (SalicylAzoSulphaPyridine; SASP)

SASP is commonly used to treat both RA [McConkey et al. 1980] and UC [Baron et al. 1962]. In the case of UC, the therapeutic efficacy of this drug is related to the 5-AminoSalicylic Acid (5-ASA) moiety, which appears to act topically [Campieri et al. 1985]. The SulphaPyridine (SP) moiety acts as a carrier of 5-ASA [Azad Khan et al. 1977] and most of the side-effects that occur in approximately one-third of the patients with UC receiving SASP are caused by SP. In attempt to reduce the high incidence of such side-effects and, at the same time reduce the absorption of 5-ASA in the small intestine, several slow- or delayed-release preparations of pure 5-ASA have been developed.

In patients with UC, 5-ASA/SASP is usually administrated in order to prevent relapses, but this drug has also been proven to reduce mild and moderate attacks of this disease. In the case of patients with RA, the active portion of the SASP molecule has been claimed to be the SP moiety [Pullar et al. 1985].

Pharmacokinetics and metabolism. SASP is absorbed to a relatively limited extent in the small intestine; 75 - 90% of the dose administrated enters the colon [Das et al. 1979]. In the colon SASP is cleaved by bacterial reductases to yield equal amounts of 5-ASA and SP [Peppercorn & Goldman 1972, Schröder 1973]. Moreover, at least in rats azo reduction of SASP appears to occur to a very minor degree, even in the absence of an intestinal microflora [Benno et al. 1996b].

Molecular aspects of the therapeutic action. The mode of action of SASP in patients with UC or RA remains unclear. Although the potential antimicrobial activity of SASP could be due either to the intact molecule and/or to its metabolites, this activity is generally attributed to the SP moiety of the molecule, which acts as a competitive inhibitor of the substrate paraaminobenzoic acid in connection with the bacterial synthesis of folic acid [Woods 1940]. This antibacterial action requires the presence of a free amino group on the aromatic ring, which is produced by cleavage of the azo bond, with release of SP. The *in vitro* antimicrobial effects ascribed to the intact SASP molecule are questionable, since the bacterial species examined under these conditions also have the capacity to cleave the azo bond to produce 5-ASA and SP [Azad Khan et al. 1983].

Studies on patients with UC have revealed no consistent effect of SASP on their microflora [Cooke 1969, West et al. 1974, Hazenberg et al. 1982,

Greenfield et al. 1983, Burke & Axon 1988]. *In vitro,* SASP, SP and 5-ASA inhibit the growth of anaerobic strains, in particular strains of *Cl. difficile* [Sandberg-Gertzén et al. 1985]. Isolated reports on patients with RA indicate that SASP inhibits the growth of *E. coli*, *Cl. perfringens* [Neumann et al. 1987] and *Cl. perfringens* [Bradley et al. 1993], as well as reducing the entire faecal content of aerobic flora, *E. coli* and *Bacteroides* [Kanerud et al. 1994].

In cultured rectal mucosa from patients suffering from active UC, SASP and 5-ASA both potently inhibit the accumulation of prostacyclin I_2, thromboxane A_2 and prostaglandin E_2, while SP inhibits only prostaglandin E_2 and prostacyclin I_2 [Ligumsky et al. 1981]. In patients with active UC who respond to treatment, administration of 5-ASA reduces the luminal levels of PGE_2 and LTB_4 in patients with active UC, who respond to the treatment [Lauritsen et al. 1986]. Moreover, *in vitro* experiments have shown that 5-ASA and, in particular, SASP block the synthesis of the products of lipo-oxygenase [Stenson & Lobos 1982].

Furthermore, SASP and 5-ASA are potential scavengers of reactive oxygen species, at least *in vitro* [Verspaget et al. 1991]. Indeed, the concentration of 5-ASA that can accumulate in the inflamed mucosa of patients with UC might be in the range required to scavenge free radicals [Grisham 1994]. In addition, treatment with 5-ASA reduces the elevated concentrations of lipid peroxides observed in the colonic mucosa of patients with UC to normal levels [Nielsen & Ahnfelt-Rønne 1991].

In an experimental model with conventional rats we found that SASP, SP and 5-ASA had a selective compartment-dependent proliferative actions on the epithelium of the intestinal tract [Benno et al. 1997].

Activation of the Peroxisome Proliferators-Activated Receptor-γ (PPAR-**γ**), a nuclear receptor expressed at relatively high levels in the colon, inhibits the production of inflammatory cytokines and chemokines, as well as the proliferation of inflammatory cells. Recently, 5-ASA was shown both to be a ligand for this receptor in the epithelial cells of the colon and to efficiently attenuate intestinal inflammation in experimental animals. Accordingly, activation of PPAR-**γ**, by 5-ASA has been proposed to be the major mechanism underlying the anti-inflammatory effects of this substance in patients with UC. Interestingly, comparision of the consequences of overexpression of this receptor in GF mice and mice with conventional intestinal flora indicated that the flora plays a pivotal role in modulating the inflammatory epithelial response [Dubuquoy et al. 2006]. Whether this is also the case for patients with RA is still unknown.

3 AIMS OF THE STUDY

The investigations described here were designed to achieve the following:

1. To establish and evaluate reference values of three major MACs in the GI microflora in healthy subjects by estimating the conversion of cholesterol to coprostanol (I), excretion of urobilinogens and tryptic activity (FTA) in faecal samples (II).

2. To characterize these MACs in patients with UC and the possible relationship of these functions to the extension of the disease (III).

3. The possible influence of SASP on these MACs in patients with RA (IV).

4 MATERIALS AND METHODS

4.1 Healthy subjects

Faecal samples from 633 healthy volunteers living in Norway, Scotland and Sweden were collected for determination of excretion of cholesterol, coprostanol, urobilinogens and tryptic activity. In case of shortage of faecal material the conversion of cholesterol to coprostanol was analysed first, followed by FTA and then urobilinogens. All of these subjects were in good health, as assessed on the basis of their medical histories and a physical examination. None of them had taken any antimicrobial drug during the period of at least 6 weeks prior to sampling and all were on a Western diet.

4.1.1   Conversion of cholesterol to coprostanol (Paper I)

In this study faecal samples from 633 healthy subjects were investigated with the following gender- and age-distribution:
334 Norwegians (133 men & 201 women; 18-43 years),
93 Scotchmen (29 men & 64 women; 18-58 years),
206 Swedes (124 men & 82 women; 21-81 years).

4.1.2   Formation of urobilinogens and degradation of tryptic activity (Paper II)

In this study faecal samples from 562 and 573 healthy subjects were investigated with respect to urobilinogens and FTA, respectively. The gender- and age-distribution were as follows:
Urobilinogens;   316 Norwegians (130 men & 186 women; age 18-43 years),
                  93 Scotchmen (29 men & 64 women; 18-58 years),
               153 Swedes (95 men & 58 women; 21-81 years).
FTA;   323 Norwegians (126 men & 197 women; 18-43 years),
                  93 Scotchmen (29 men & 64 women; 18-58 years),
               157 Swedes (105 men & 52 women; 21-81 years).

 For analysis, this entire population was divided into three arbitrary different age groups, i.e., <36 years, 36-50 years and >50 years old.

4.2   Patients with ulcerative colitis (Paper III)

A total of 42 patients exhibiting either clinically and biochemically inactive proctitis (n=7) or left-sided (n=18) or total UC (n=17) were included in this study. The extent of the disease was determined on the basis of rectosigmoideoscopy/colonoscopy and all demonstrated histological changes indicative of UC. Patients with mucosal changes proximal to the hepatic or distal to the splenic flexures were considered to exhibit total or left-sided UC, respectively. They had been given a daily median dose of 2 g SASP for at least 3 months before the faecal sampling.

4.3   Patients with rheumatoid arthritis (Paper IV)

This study involved 40 patients with RA, as diagnosed on the basis of the Revised Criteria of the American Rheumatism Association (Arnett et al. 1988), all of whom had rheumatoid factor in their serum, together with symmetrical erosive joint disease. Of these, 19 were on continuous treatment with SASP (at a median daily dose of 2 g, range 2-3 g) for at least 3 months prior to faecal sampling. Intake of <u>N</u>on-<u>S</u>teroidal <u>A</u>nti-<u>i</u>nflammatory <u>D</u>rugs (NSAIDs) was reported by 16 patients taking SASP and 14 of the 21 patients not taking SASP. At the time when the faecal samples were collected, the functional capacity of these patients was assessed employing a health questionnaire (HAQ) along with biochemical tests for the presence of inflammation.

4.4   Healthy controls (Papers III & IV)

In Paper III, the patients with UC were compared with 42 volunteers matched for gender and age.

In the case of Paper IV, the patients with RA who were not receiving SASP were compared with 21 volunteers matched for gender and age.

None of these control subjects had any history of GI disorders and all were also included in Papers I and II.

Table III. Demographic characteristics of the patients with ulcerative procto-/colitis (Paper III) or rheumatoid arthritis (Paper IV) at the time of faecal sampling.

Disease	n[a]	Sex	Age[b]	Duration of the disease[b]
		(M/F)	(years)	(years)
Ulcerative proctitis	7	4/3	25 (23-37)	5 (3-10)
Left-sided colitis	18	10/8	41 (36-48)	5 (3-10)
Total colitis	17	11/6	44 (38-52)	11 (5-17)
Rheumatoid arthritis without treatment with SASP[c]	21	5/16	61 (55-65)	8 (6-16)
Rheumatoid arthritis with SASP[c] treatment	19	7/12	55 (45-69)	11 (3-14)

[a] = number of subjects
[b] = values are medians and interquartile ranges (25th-75th percentiles)
[c] = salicylazosulphapyridine (sulphasalazine)

4.5   Collection of faecal samples

Faecal samples were collected in plastic vials and frozen at -20^0 C within five minutes after collection. For analysis, the samples were thawed at room temperature and homogenized manually. Aliquot was transferred to a plastic vial for determination of urobilinogens and another aliquot was mixed with saline 1:2 (w/w), incubated at $+4^0$C for 2 h and thereafter centrifuged (35 000 x g at $+4^0$C for 30 min). Aliquots of the supernatant were used for determination of FTA. The combined supernatant and sediment was used for determination of cholesterol and coprostanol.

4.6   Determination of cholesterol and coprostanol

 The combined supernatant and sediment, obtained as described above, were mixed with a solution of 95% ethanol and 10 M NaOH (2:1) and hydrolysed at 60^0 C for 45 min, extracted twice with 10 ml hexane, and thereafter with 70% ethanol until neutral pH was attained. The cholesterol and coprostanol were separated by gas-liquid chromatography (Pye-Unicam GCD-104) at 290^0C on 3% OV-17 (Supelco, USA) [Midtvedt et al. 1990]. Using this method, peak areas < 5% were ignored as impurities [Gustafsson & Werner 1968].
    The results were expressed as the percentage of coprostanol in the total amount of cholesterol and coprostanol present.

For purposes of classification, subjects were divided in three groups, non converters with a conversion rate of < 5%, low converters with a conversion rate ≥ 5% but < 40% and high converters with a conversion rate ≥ 40%.

4.7   Determination of urobilinogens

For measurement of urobilinogens, 0.7 g aliquots of faecal material were homogenized in 4.2 ml distilled water utilizing an Ultra-Turrax homogenizer. Subsequently, 4.7 ml of 1.3 M ferrous sulphate, and 4.7 ml of 2.5 M sodium hydroxide were added, and the resulting suspension was allowed to stand in the dark at room temperature for 2 h. Then this preparation was filtered; the filtrate thus obtained was centrifuged at 35 000 x g for 20 min at 4^0C; and 2.0-ml aliquots of the resulting supernatant were used as samples and blank solutions, respectively. The absorbance of these samples and blanks at 562 nm against distilled water in the reference cuvette was determined with a Hitachi 150-20 spectrophotometer. Phenolsulphonphthalein was used as an artificial standard for "urobilinogen-aldehyde". Finally, the values thus obtained were converted to mmol/kg faeces (wet weight) using 596 Da as the molecular weight for urobilinogen [Saxerholt 1990].

4.8   Determination of tryptic activity

For determination of FTA 0.1 ml of supernatant was added to 2.9 ml Tris buffer, pH 8.2, containing 4.4 g calcium chloride per liter. Thereafter, 0.6 ml 0.003 M BAPNA (N-benzoyl-DL-arginine-paranitroanilide hydrochloride) (Sigma, St Louise, MO, USA) was added as substrate and the reaction was carried out at room temperature for 10 min and stopped by adding 0.6 ml 5 M acetic acid. For preparation of the standard curve, bovine pancreas trypsin (type III, Sigma) diluted in 2 mM hydrochloric acid was used. Each sample was analysed in parallel with a blank containing the same amount of sample, but no substrate, and the readings were performed on a Hitachi 150-20 spectrophotometer at 405 nm. On the basis of comparision with the standard curve the enzyme activity was finally expressed as mg tryptic activity /kg wet weight faeces [Norin et al. 1988].

4.9   Statistical analysis

All tests were two-sided and p<0.05 were considered to be statistically significant.

Paper I

The prevalences of non-converters, low-converters and high-converters in Norway, Scotland and Sweden were first compared by factorial ANOVA, employing country, gender and age group as the factors. Since this analysis revealed no significant differences between the countries, the data for each group from all three countries were pooled to obtain reference values. The homogeneity of the group variances was analysed pair-wise by the Welch t-test, with separate estimates of variance.

Paper II

Differences between urobilinogens and FTA obtained for the three countries were first subjected to statistical analysis using both the chi-square test and Kruskal-Wallis (ANOVA). Results from these analyses revealed no statistically significant differences, and subsequently data for men and women by age group were pooled and possible differences examined employing the procedure for least significant difference (LSD).

Papers III and IV

Data were present as medians and interquartile ranges (25th-75th percentiles). The Kruskal-Wallis and Mann-Whitney non-parametric tests and the chi-square test were used for between-group analyses.

5 RESULTS

5.1 Healthy subjects (Papers I and II)

As mentioned above, since no significant differences between our healthy subjects living in Norway, Scotland and Sweden were observed, the data from these three countries were pooled.

5.1.1 Conversion of cholesterol to coprostanol (Paper I)

For purposes of classification, subjects were divided in non-converters (<5% conversion rate), low converters (between 5% and < 40% conversion rate) and high converters (≥40% conversion rate). Among men, the proportion of non-converters was higher in the youngest than in the two older groups (17% versus 7% and 2% in the groups of 36-50 and >50-year-olds, respectively; p<0.05); while the percentage of high converters was highest in the oldest age group (84% versus 62% and 60% in <36 and 36-50-year-olds, respectively; p<0.05). In females, the distribution of subjects over categories non-, low- and high converters were similar in all three age groups. The proportion of high converters among women younger than 36 was significantly higher than among men in the same age-group.
No statistically significant increase in mean values was found in any group (see Figure 1 in Paper I).

5.1.2 Formation of urobilinogens and degradation of tryptic activity (Paper II)

The faecal levels of urobilinogens were significantly higher in men in all age groups (1.31, 1.13 and 1.37 versus 1.03, 0.89 and 0.99 mmol/kg faeces in men and women, respectively). In 36-50-year-old men, the mean level of urobilinogens was significantly lower than for men in the other two age groups. Moreover, women in the youngest age-group had significantly higher urobilinogen levels than those 36-50 years of age. In addition, men younger than 36 years of age demonstrated significantly higher activity than the older men. The levels of FTA were higher in men than in the female subjects, significantly so in the case of the two younger age groups (192, 105 and 50 versus 60, 53 and 39 mg/kg faeces in <36-, 36-50- and >50-year-old men and women, respectively).

5.2 Patients with ulcerative colitis (Paper III)

No significant differences in MACs were detected in patients with proctitis or more extensive UC, subsequently the values for these groups were pooled and for comparision to those of gender- and age-matched healthy controls.

5.2.1 Conversion of cholesterol to coprostanol

The median value in UC patients was 25% (0-69%), which was significantly lower than the 50% (35-90%) obtained for the control subjects (p<0.05). (Presented in Paper III, Table I and Figure 1a). Twenty-four (57.1%) of the 42 patients converted less than 40% of their cholesterol to coprostanol and were therefore classified as low converters, versus 33.3% (18/42), among the healthy subjects (p<0.05) (Table IV).

5.2.2 Formation of urobilinogens (Table V)

Patients exhibited a median value of 0.3 (0.2-0.6) mmol urobilinogens/kg faeces, which was significantly lower than the control value of 1.1 (0.7-1.5) mmol/kg faeces.

5.2.3 Degradation of tryptic activity (Table V)

The median value of 291 (82-487) mg /kg faeces, observed in the patients was markedly higher than the control value of 20 (0-104) mg/kg faeces (p<0.001).

5.3 Patients with rheumatoid arthritis (Paper IV)

5.3.1 Conversion of cholesterol to coprostanol (Table IV)

One of the 21 (4.8%) of the patients not being treated with SASP were low converter, while of the 7 of the 21 (33.3%) of healthy subjects fell into this category (p<0.05). Three of the 19 (15.8%) patients administrated SASP were low converters (p>0.05 versus untreated patients with RA).

5.3.2 Formation of urobilinogens (Table V)

The median faecal level of urobilinogens for patients not receiving SASP treatment was 1.1 (0.6-1.4) mmol/kg faeces, which was significantly higher than the corresponding value for those patients taking this drug (0.5 (0.3-0.7) mmol/kg faeces).

5.3.3 Degradation of tryptic activity (Table V)

The median FTA of 21 (0-53) mg/kg faeces observed in patients not on SASP, was significantly lower than the corresponding value for patients receiving this drug (i.e., 237 (86-394) mg/kg faeces).

Table IV. The subject distribution of low and high converters of cholesterol to coprostanol among patients with ulcerative colitis taking sulphasalazine (SASP) (I), patients with rheumatoid arthritis who were or were not being treated with this drug (II) and healthy age- and sex-matched subjects (controls in I and II, respectively).

Group (n[a])	Low converters (%)	High converters (%)
Controls in I (42)	33.3	66.7
Patients with ulcerative colitis given SASP° (42)	57.1[#]	42.9
Controls in II (21)	33.3	66.7
Patients with rheumatoid arthritis not given SASP (21)	4.8*	95.2
Patients with rheumatoid arthritis given SASP (19)	15.8	84.2

Subjects exhibiting a faecal value for $[(\text{coprostanol\%} = \text{coprostanol}/(\text{cholesterol} + \text{coprostanol}) \times 100(\%)]$ of $<$ or $\geq 40\%$ were defined as low and high converters, respectively.

[a] = number of subjects

[#] = $p < 0.05$ compared to the controls in I

* = $p < 0.05$, compared to the controls in II

° = samples pooled from patients with proctitis and more extensive colitis

Table V. Faecal levels of urobilinogens and tryptic activity (FTA) among patients with ulcerative colitis who were taking sulphasalazine (SASP), patients with rheumatoid arthritis who were or were not being treated with this drug (II) and healthy gender- and age-matched subjects (controls I and II, respectively). The values presented are medians (25th-75th percentiles).

Group (n[a])	Urobilinogens (mmol/kg faeces)	FTA (mg/kg faeces)
Controls in I (42)	1.1 (0.7-1.5)	20 (0-104)
Patients with ulcerative colitis given SASP (42)	0.3 (0.2-0.6)[¶]	291 (82-487)[¶]
Controls in II (21)	1.1 (0.6-1.3)	0 (0-30)
Patients with rheumatoid arthritis not given SASP (21)	1.1 (0.6-1.4)	21 (0-53)
Patients with rheumatoid given SASP (19)	0.5* (0.3-0.7)	237* (86-394)

[a] = number of subjects

[¶] = $p < 0.05$ compared to the healthy controls in I

* = $p < 0.05$ compared to patients with rheumatoid arthritis not taking SASP

6 DISCUSSION

In the present investigation we have established reference values for three MACs - i.e. conversion of cholesterol to coprostanol, levels of urobilinogens and FTA – in more than 560 healthy subjects and compared these to the corresponding values in patients with UC (Paper III) or RA (Paper IV). This approach is highly suitable for evaluating the sum of the changing and broad interactions that occur between the microflora, the intraluminal environment and the host including biochemical, immunological and physiological factors. The other approaches to such an evaluation described above involve the obvious limitations associated with *in vitro* studies or imposed by the complexity of bacterial-host interactions, which restricts the sensitivity and specificity of most indirect *in vivo* tests.

In this context it is important to emphasize that alterations in MACs observed under various conditions may reflect an influence on the host and/or the microbial flora. Although MACs can be measured directly, in many cases interpretation of these measurements requires an indirect evaluation of the host response (as described previously). At the same time, evaluation of MACs can provide information about specific bacteria and/or bacterial functions, as well as on the specific compartments in the GI tract in which the interactions occur, as exemplified by the reduction of cholesterol to coprostanol in caecectomized animals [Kellogg 1973].

6.1 Conversion of cholesterol to coprostanol

6.1.1 Healthy subjects

Previous studies on humans have revealed that microflora which can reduce cholesterol are established in the GI tract during the second half of the first year of life [Midtvedt & Midtvedt 1993] and remain essentially stable thereafter [Wilkins & Hackman 1974]. Interestingly, breast-feeding appears to delay the development of this metabolic function [Midtvedt et al. 1988].

Among healthy adult individuals the intestinal capacity for degradation of cholesterol varies widely [Reddy & Wynder 1973, Reddy et al. 1977, Lipkin et al. 1981]. When Wilkins and Hackman [1974] evaluated the converting capacity by determining the frequency distribution of the percentage of cholesterol metabolised in healthy subjects they found a bimodal distribution, in which most people convert a high percentage of intestinal cholesterol to coprostanol, whereas others convert much less. Since then, employing arbitrary limits between high and low conversion of 40-60%, several other investigations have confirmed this bimodal distribution of intestinal cholesterol-reducing [Salyers et al. 1977, Lipkin et al. 1981, Korpela & Adlercreutz 1985, Midtvedt et al. 1990].

In some of these studies, gender and age were also taken into consideration [Wilkins and Hackman 1974, Salyers et al. 1977, Korpela & Adlercreutz 1985], but these factors appeared to exert no significant influence. However, as

documented in Table II, Paper I, the largest number of subjects so far presented in a single study was 108 [Wilkins and Hackman 1974, Salyers et al. 1977, Midtvedt & Frederichsen 1977, Lipkin et al. 1981, Korpela et al. 1984, Korpela & Adlercreutz 1985, Midtvedt et al. 1990, Benno et al. 1994]. Thus, the large number of subjects involved in our present studies give the results considerably more power.

The simple fact that this function is performed only by a small proportion of the intestinal flora may render it especielly vulnerable to alterations in the intestinal environment. On the basis of *in vitro* findings, Sadzikowski and coworkers [1977] proposed that the differences in the amount of coprostanol in faeces of individuals may be due to different susceptibilities of these microorganisms to certain antagonistic intestinal bacteria (the inhibition hypothesis). We reported previously that formation of coprostanol in man [Midtved & Fredrichsen 1977] as well as in animals [Carlstedt-Duke et al. 1985, Gustafsson & Norin 1977] is significantly, but temporarily, reduced by administration of several antibiotics. However, since no investigation of antibiotic susceptibility among coprostanol-forming strains has been carried out *per se*, the observed effects might be due to an effect upon other members of the intestinal flora. Interestingly, there are some few observations demonstrating a switch from a lower to a higher conversion following ingestion of antibiotics [Midtvedt & Frederichsen 1977]. Indirectly these observation strengthen the inhibition hypothesis.

The so-called receptor hypothesis provides an alternative explanation for individual differences in faecal levels of coprostanol [Karlsson et al. 1991]. It can be assumed that the stable establishment of a non-motile, non-spore-forming and strictly anaerobic microorganism in relatively low numbers in certain intestinal compartments might involve adhesion to the mucosal surface via specific receptors. Indeed, it has been postulated that several microbial species may require two different types of receptors [Karlsson 1989, Karlsson et al. 1991]. According to this proposal the first-step receptors mediate host and tissue tropism, but do not establish a stable proximity to the host cell. However, a binding to second-step, low-affinity receptor achieves this close proximity and, furthermore this binding might be susceptible to inhibition by neighbouring structures.

As described above, formation of coprostanol in infants commences in the second half of the first year of life [Midtvedt & Midtvedt 1993] and our hypothesis is that genetic factors may be involved, possibly in the form of presentation of second-step receptors, which need to be unmasked. Complete blockage of the second-step receptors by neighbouring structures or total lack of expression of the first-step receptors might explain the existence of non-converters. The switch from non- to high conversion observed following antibiotic therapy (as described previously) could then be due to temporary unmasking of second-step receptors. Similarly, antibiotics might eradicate microbes capable of unmasking second-step receptors. To date, however, the nature of the postulated receptor(s) remains unknown. Moreover, the inhibition

24

and receptor hypotheses are not mutually exclusive but may both be involved, simultaneously or under different conditions, as in complex biofilms, establishing a stable cholesterol reducing microbiota.

A key question is why the proportions of non- and low converters are reduced in the oldest group of healthy men that we studied. As pointed out, little or no converting capacity for intestinal metabolism could be a risk factor for morbidity. Over the years attempts have been made to relate the conversion of cholesterol to coprostanol to certain forms of intestinal disease. For instance, individuals with familial colon polyposis or familial colon cancer have elevated faecal cholesterol, with relatively little conversion to coprostanol [Lipkin et al. 1981]. In addition to these inherited forms of diseases, the incidence of colon cancer varies geographically, being low in Asia, South America and Africa and high in areas such as northwestern Europe and North America, where dietary intake of animal protein and fat is relatively high which is usually referred to as a "Western diet". In 1971 Hill and Aries reported that faecal levels of neutral steroids (including cholesterol) in English and Scottish individuals were higher than these observed for Ugandans and Indians and, in addition, these faecal steroids were more extensively metabolized in the case of the Europeans.

Two years later Reddy and Wynder [1973] reported that in the faeces of individuals living in areas where the incidence of colon cancer is low (Japanese and Chinese), a lower percentage of the cholesterol present has been metabolized in comparision to Americans. Moreover, in a clinical study in 1987 Peuchant and coworkers hypothesized that high levels of coprostanol exhibit a relationship to malignancy on colon cells. However, the discrepancy between the findings in familiar studies and non-familiar form of colon cancer remains to be elucidated.

Additionally, it should be underlined that the results obtained in our study, i.e. a high conversion rate of cholesterol to coprostanol in elderly healthy men, do not support the assumption that coprostanol is a primary risk factor for development of colon cancer.

In addition to being such a risk factor for colon cancer, it has been proposed that easy reabsorption of unmetabolized cholesterol might influence circulating levels of this steroid. Indeed, the serum level of cholesterol in GF rats is higher than in their conventional counterparts fed the same diet [Danielsson & Gustafsson 1959] and, moreover, oral administration of cholesterol-reducing bacteria to GF mice reduces their serum level of cholesterol [Li et al. 1998]. From a functional point of view, conversion of cholesterol to coprostanol by the intestinal flora can be looked upon as a sharp "microbial intestinal knife", influencing the normal enterohepatic circulation of cholesterol and consequently the risk for developing artheriosclerotic disease [Midtvedt 1987]. In the one clinical investigation designed to examine this possibility, no correlation between the extent of intestinal metabolism of cholesterol and levels of serum cholesterol could be demonstrated, but only five subjects were involved [Korpela et al. 1984].

Our present findings provide no support for a continous increase in the extent of conversion of cholesterol to coprostanol with increasing age. Therefore, in agreement with others (as described previously), we conclude that once established this microbial function is essentially stable by time. However, if a non- or low intestinal conversion of cholesterol to coprostanol is a morbidity factor, individuals with such conversion may be selectively excluded from the healthy population with increasing age, and this may have influenced our findings.

6.1.2   Patients with ulcerative colitis

Irrespective of whether the conversion of cholesterol to coprostanol was expressed as the median values for all patients (Paper III, Figure 1a, Table II) or divided into groups with different converting capacities (Papers I, Table I; Paper IV, Table II), we found that patients with UC exhibit significantly less formation of coprostanol. This reduction was unrelated to the limited or more wide-spread localization of the disease and our patients demonstrated no evidence of an ongoing inflammation, or clinically detectable disturbances in intestinal motility or any other changes that might facilitate bacterial overgrowth. Together, these observations indicate that the cholesterol-reducing capacity of the intestinal flora in UC patients is reduced. This conclusion is in agreement with Reddy and coworkers [1977], who found that patients with UC excrete higher amounts of neutral sterols (including cholesterol), but relatively less coprostanol. These authors stated that their patients were not receiving antibiotic treatment, but did not specify whether or not they were taking SASP.

6.1.3   Patients with rheumatoid arthritis

In comparision to gender- and age- matched control subjects, significantly more of the patients with RA who were not receiving SASP treatment were high converters indicating that the numbers and/or location of these patients´ coprostanol-producing microbes differs from that of the normal population. Interestingly, fragments of the cell-wall of anaerobic Gram-positive rods, among others *Eubacterium lentum* (i.e., a species known to be involved in formation of coprostanol) - isolated from the human intestine, have peptidoglycan that can induce severe and chronic arthritis in rats [Hazenberg et al. 1992]. Since antibodies to the faecal peptidoglycan-polysaccharide complexes have been detected in the serum of the patients with RA, the immune system of these patients must have experienced contact with the peptidoglycan-polysaccharide molecule. Furthermore, such fragments are present on macrophages in the spleen of patients, indicating clearly that they can pass through the intestinal wall [Hazenberg et al. 1992]. These findings fit with our own results.

6.2 Formation of urobilinogens

6.2.1 Healthy subjects

All of our subjects excreted urobilinogens, indicating that this MAC reflects a basic host- flora interaction. The significant gender differences observed in this respect within all age groups could reflect several different and partially counteracting factors: 1) In general, the total numbers of erythrocytes are lower in females [Fairbanks et al. 1996], which results in less biliary excretion of bilirubin. 2) The longer intestinal transit time demonstrated by women [Meier et al. 1995] may allow more extensive microbial transformation of bilirubin to urobilinogens. 3) Higher faecal mass in men tends to lower the levels of compounds present [Hill 1971].

6.2.2 Patients with ulcerative colitis

Irrespective of how widespread the disease was, patients with UC excreted significantly lower amounts of urobilinogens, demonstrating alterations in this particular metabolic interaction between the intestinal flora and the host. For further discussion, see under headline "Influence of SASP and NSAIDs" below.

6.2.3 Patients with rheumatoid arthritis

In contrast, the excretion of urobilinogens by patients with RA not receiving SASP treatment was similar to that of healthy individuals, indicating a lack of any major change in this function of the intestinal flora in this group of patients. For further discussion, see under headline "Influence of SASP and NSAIDs" below.

6.3 Degradation of tryptic activity

6.3.1 Healthy subjects

As mentioned previously, FTA is the net sum of complex interactions between pancreatic trypsinogen and host/diet/microbially-derived activators and inactivators. In principle, a relatively high intake of protein in combination with unaltered colonic transit time and unchanged production of PSTI could result in an elevated level of FTA. Unfortunately, in the present investigation the dietary profiles of our subjects were not evaluated routinely. However, dietary surveys in all of the three countries involved revealed that at all ages men ingest larger quantities of protein than do women [Riksmaten 1997-98, Norkost 1999, The Scottish health survey 1998]. FTA has previously been examined in healthy individuals consuming a Western diet, with pronounced interindividual variations [Norin et al. 1988]. In addition, GF rats and mice exhibit high levels of FTA, whereas the corresponding level in their

conventional counterparts is consistently zero [Norin et al. 1986a, Norin et al. 1986b].

Moreover, in general men secrete higher levels of pancreatic enzymes [Gaia et al. 1984] and in both sexes this secretion decline with increasing age [Laugier et al. 1991]. However, in neither of these studies was trypsin determined specifically. In addition, the colonic transit time in females of all ages is longer than males of the same age [Meier et al. 1995]. Together, these factors may explain for the gender differences observed here.

6.3.2   Patients with ulcerative colitis

Significantly elevated levels of FTA demonstrated by patients with UC reflect disturbances in the metabolic activity of their intestinal flora.

The procedure commonly employed for the assay of tryptic activity also measures the activity of tryptase, another serine protease. Interestingly, this latter enzyme is stored exclusively in the secretory granules of mast cells and, furthermore, the number of mast cells in the intestinal mucosa of patients with UC and active inflammation is elevated markedly and a significant increase in the secretion of tryptase from the mucosa of these patients has been reported [Raithel et al. 2001]. However, none of our patients exhibited any signs of active intestinal inflammation indicating that there were probably no alterations in tryptase activity, which might have influenced our findings.

6.3.3   Patients with rheumatoid arthritis

Our observations that the FTA values of patients with RA were similar to those of healthy control individuals indicates that there are no major alterations in the capacity of the intestinal flora in such patients to degrade trypsin.

6.4   The influence of environmental factors on MACs

6.4.1   Diet

The influence of diet on the capacity to reduce cholesterol capacity has been the subject of considerable debate. Early studies by Wells and Cooper [1958] and Kellogg and Wostmann [1966] indicated that a diet containing large amounts of carbohydrates that are incompletely absorbed may suppress the formation of coprostanol. This conclusion is supported by the observation that in gnotobiotic rats colonized by a coprostanol-producing microflora, replacement of dietary starch by lactose reduces the number of these microorganisms, with a consequent reduction of more than 70% in coprostanol production [Eyssen & Parmentier 1974]. Dietary surveys from the three countries involved in our investigations [The Scottish health survey 1998, Norkost 1999, Riksmaten 2002] reveal both that young men consume more milk than older men and that men in general consume more milk than women.

Determination of whether such gender- and/or age- related differences in dietary intake of lactose may account, at least in part, for the differences observed here in the transformation of cholesterol to coprostanol is beyond the scope of the present study.

The fact that vegetarians and omnivores exhibit the same pattern of conversion of cholesterol to coprostanol strongly indicates that the level of dietary intake of fiber does not influence this microbial function [Korpela & Adlercreutz 1985, Norin et al. 1998]. Moreover, reevaluation of the data reported by Reddy and coworkers [1992] supports the conclusion that diet has little, if any impact, on this conversion. However, as already mentioned, we have no specific dietary records for our subjects.

A dietary trial in which 53 patients with RA either fasted or consumed a vegan/vegetarian diet revealed significant changes in the intestinal flora of those patients who demonstrated clinical improvement [Peltonen et al. 1994], but no differences in the formation of coprostanol and degradation of FTA (unpublished data).

6.4.2   Influence of SASP and NSAIDs

For ethical reasons, we could not evaluate the influence of SASP treatment of UC patients on their MAC variables (Paper III). In the case of patients with RA (Paper IV), treatment with this drug tended to reduce coprostanol formation, as well as to significantly decrease inactivation of FTA and formation of urobilinogens. Moreover, the proportion of low converters of cholesterol to coprostanol among our patients with UC and taking SASP, was significantly greater than in the control group. Indirectly, it might be reasonable to assume that the observed alterations in our MACs in patients with UC are influenced by the SASP treatment.

A variety of mechanisms may underlie such alterations caused by this drug. SP, the sulphonamide moiety of the SASP molecule, is a well known antibacterial drug and it has been claimed, although never satisfactorily proven, that this antibacterial effect is responsible for the anti-rheumatic activity of the drug [Pullar et al. 1985, Neumann et al. 1987]. However, it is noteworthy in this context that SP rapidly evokes microbial resistance [Hazenberg et al. 1982] and, in addition, does not affect the spectrum of microflora observed in connection with routine microbiological analysis [Cooke 1969]. Thus, it seems reasonable to assume that the long-term anti-rheumatic activity of SASP is not due to an effect of the flora, but are related to effect(s) on host functions. As shown above, SASP given to patients with RA cause a significant inhibition of two MACs, i.e. bilirubin and FTA as well as a reduction of the formation of coprostanol.

It is well known that SP cause a potent inhibition of human carbonic anhydrases [Supuran 2008] present in the wall of the human intestine [Parkkila et al. 1994]. This inhibition increases the flux of Na^+ and Cl^- into the intestinal

lumen, while also decreasing the intracolonic pH, thereby altering the local mucosal micro-climate and the conditions for the surface related intestinal microflora.

At the same time, administration of SASP leads to the formation of high concentrations of 5-ASA, an iron chelator, [Grisham et al. 1992], in the lumen of the colon [Azad Khan & Truelove 1982]. Intraluminal binding of iron by 5-ASA may inhibit the growth and metabolism of intestinal bacteria, thereby contributing to the alterations in MAC parameters observed in patients receiving SASP.

Yet another possibility involves the ability of SASP to scavange free radicals [Verspaget et al. 1991] and influence the local micro-climate in this respect as well. However since the patients with RA demonstrated no intestinal inflammation and those with UC were in remission, it appears unlikely that the alterations in MAC functions observed here are due to changes in the levels of free radicals.

Similar reasoning indicates that the reduction in the faecal content of urobilinogens is probably also a consequence of alterations in the intestinal micro-climate. It should be noted that hemolysis is a well-known side-effect caused by the SP moiety of SASP [van Hees et al. 1978] and a significant degree of hemolysis in our patients receiving this drug should have produced elevated levels of bilirubin for further conversion to urobilinogens. Since faecal levels of these metabolites are actually lower in these patients, we conclude that in this context the major effect of SASP on the host is inhibition of the conversion of bilirubin to urobilinogens.

The high levels of FTA present in patients with UC receiving SASP might not be due to a direct effect on the microflora, but rather, to alterations in the intestinal micro-climate or be due to alterations in the amount of PSTI. In this context, Playford and coworkers [1995] found, compared to normal subjects, reduced levels of mucosal PSTI in affected segments of the colon in patients with active or quiescent UC.

Additionally, Mukherjee and coworkers [2009] have found that antibacterial proteins produced in the intestinal mucosa and secreted into the lumen are activated by trypsin. These proteins (HIP/PAP = hepatointestinal pancreatic/pancreatitis-associated proteins) are bactericidal at low micromolar concentrations for Gram-positive bacteria and their expression are up-regulated in patients with IBD. In this context it is worth noting that intracolonic administration of trypsin to conventional mice can cause mucosal inflammation [Yoshida et al. 2006], whereas the high amounts of trypsin, normally found in the intestines in GF mice, do not give rise to any inflammation.

Taken together, the high levels of FTA in patients with UC might be due to an absence of trypsin degrading microbes, or an influence of SASP as supported by our data in patients with RA, or might be due to reduced production of PSTI, either taking place in the pancreatic gland or the intestinal tract.

For ethical reasons, a proper control group, i.e. patients not receiving SASP has not been evaluated, and the data from our UC patients with proctitis make a local influence of PSTI clearly uncertain.

Thus, the relative contributions of these factors remain unanswered.

7 SUMMARY AND CONCLUSIONS

In the present investigation we have established and evaluated reliable reference values for three Microflora-Associated Characteristics (MACs) - i.e., conversion of cholesterol to coprostanol, formation of urobilinogens and presence of Faecal Tryptic Activity (FTA) – in what is the largest study on these parameters to be reported to date. Men and women and different age groups, (i.e. < 36, 36 - 50 and > 50 years old) were analyzed separately and compared. Furthermore, these same three MACs were also examined in patients suffering from Ulcerative Colitis (UC) or Rheumatoid Arthritis (RA).

Conversion of cholesterol to coprostanol in 633 healthy subjects
For purposes of classification, these subjects were divided into non-, low and high converters, with conversion rates of < 5%, 5-<40% and ≥40%, respectively.

   In general, in men the percentage of individuals belonging to the groups non- and low converters was reduced by age, whereas the women of all ages exhibited similar levels of conversion. Moreover, the proportion of high converters among women younger than 36 was significantly higher than among men in the same age group. One possible explanation for these findings might be that males with non- and low converting capacity in the oldest age group are lost in this cohort of healthy subjects. However, to rule out whether the converting capacity of cholesterol to coprostanol is a morbidity factor requires further long-term studies.

Formation of urobilinogens in 562 healthy subjects
In general, in 36-50-year-old men the level was lower compared with the two other age groups of the same gender. In the case of women, the highest corresponding values were observed in the youngest age group. In all three age groups the levels were higher in men.

   These gender differences probably reflect at least three gender-related factors: erythrocytes turn over more slowly in women (resulting in less biliary excretion of bilirubin); women exhibit a longer intestinal transit time (thereby allowing more extensive microbial degradation); while the lower faecal mass in women tends to increase the concentration of urobilinogens, thus counteracting the first two of these factors.

   Bilirubin and its urobilinogen metabolites provide an interesting endogenous model for xenobiotics which also undergo enterohepatic circulation. In this context, the reference values documented here might be useful in predicting the excretion of foreign compounds in relationship to gender and age.

Degradation of tryptic activity in 573 healthy subjects
In general, the mean levels were highest in the youngest group of men and decreased in the two older age groups, significantly so between men younger

than 36 years of age and the group of men of 36-50 years of age. The corresponding value for women was also highest in the youngest group, but in this case none of the differences between age groups was statistically significant. At all ages, men had higher mean values than women, significantly higher in the case of the two younger age groups.

The alterations described here reflect numerous complex interactions between the flora, host and exogenous factors, including higher intake of protein and greater secretion of pancreatic enzymes by younger individuals of both sexes and by men in comparison to women of the same age. The slower colonic transit exhibited by women may also have exerted an influence on our findings.

FTA is probably an important regulator of the intestinal flora by its activation of secreted antibacterial proteins. The clinical relevance of our findings in patients with inflammatory bowel syndrome should be explored in greater details.

Examination of these same MACs in two different groups of patients

Patients suffering from UC;
Regardless of the extent to which their disease had spread, patients with UC demonstrated significantly less formation of coprostanol, decreased levels of urobilinogens, elevated levels of FTA in comparison to healthy subjects. Since it was impossible for ethical reasons to assess the influence of SalicylAzoSulphaPyridine (SASP; sulphasalazine) on these patients, individuals suffering from RA and receiving or not receiving treatment with SASP were also examined.

Patients suffering from RA;
Among those patients with RA who were not being treated with SASP the proportion exhibiting high conversion of cholesterol to coprostanol was higher than among the healthy control subjects; but, at the same time, there were no differences in the levels of urobilinogens and FTA.

Treatment with SASP tended to be associated with a reduction in the formation of coprostanol, along with a pronounced decrease in the faecal content of urobilinogens and an increase in FTA in comparison to the patients to whom this drug was not being administrated. Thus, it seems reasonable to assume that the alterations observed in our patients with UC were also induced by treatment with SASP, which appears to exert a general influence on the cross-talk between different types of microbes and between microbes and the host that is going on continuously in the gastrointestinal tract.

From these findings we draw the following conclusions:
i. patients with RA have elevated levels of coprostanol formation,
ii. SASP treatment induces inhibition of all three MACs.

8 ACKNOWLEDGEMENTS

I began the research described here some time ago. Ten years after starting, I received my "licentiatexamen", which was designated as a "half-time control" according to the rules then applied by Karolinska Institutet. A highly personal interpretation of "half-time control" was that I could continue doing research for another ten years in order to earn my doctoral degree, which allowed me the opportunity and value advantage of maintaining long-term contact with the following individuals, to all of whom I wish to express my gratitude:

Professor *Tore Midtvedt*, my scientific advisor, who inspired me throughout these years with warmth and humour and excellent knowledge in the field of microbial ecology, as well as numerous enjoyable discussions about other important aspects of life.

Associate Professor *Elisabeth Norin*, my second scientific advisor, for a warm friendship, for valuable scientific discussions, in particular concerning methodology, and, last but not least, for helping me with all the formalities and forms associated with this work.

Associate Professor *Andrés Uribe*, my third scientific advisor, for his burning enthusiasm for me to complete my "half-time control".

My other coworkers Drs *Eje Collinder*, *Kenneth Henriksson*, *Carl-Eric Leijonmarck*, *Karsten Midtvedt* and *Ulla Monsén*, for their valuable contributions and discussions.

Anna-Karin Persson and *IngaLillPersson* for skilled technical and administrative assistance.

In addition, I want to thank many others for helping me along the way by being a valuable part of my life:

Dr. *Mahbub Alam*, Dr. *Johan Bark*, Dr. *Ragnar Befrits*, *Ingmarie Elthammar*, *Marit Hallmans*, Dr. *Per Hellström*, Dr. *Mikael Holst*, *Marianne Norén*, *Ann-Louise Storbäck*, Dr. *Kristina Zachrisson*, close friends and valued colleagues.

My cousins, *Lennart Danin* and *John Danin*, and their families, for close friendships and support in the different phases of life.

Malin, my beloved oldest daughter, *Stefan* and their wonderful "*Little Isabelle*", my granddaughter.
Frida, my beloved daughter and her *Andy*
Per Benno, my brother and his family
Monica, without your love, support and care, I would never have made it.
My mother and late father – Dad, I wish you were here!

9 REFERENCES

Alamanos Y, Voulgari PV, Drosos AA. Incidence and prevalence of rheumatoid arthritis, based on the 1987 American college of rheumatology criteria: a systematic review. Semin Arthritis Rheum 2006;36:182-188.

Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-324.

Azad Khan AK, Piris J, Truelove SC. An experiment to determine the active therapeutic moiety of sulphasalazine. Lancet 1977;2:892-895.

Azad Khan AK & Truelove SC. The disposition and metabolism of sulphasalazine (salicylazosulphapyridine) in man. Brit J Clin Pharmacol 1982;13:523-528.

Azad Khan AK, Johnston GG, Truelove SC, et al. Tissue and bacterial splitting of sulphasalazine. Clin Sci 1983;64:349-354.

Baron JH, Connell AM, Lennard-Jones JE, et al. Sulphasalazine and salicylazasulphadimidine in ulcerative colitis. Lancet 1962;1:1094-1096.

Benno P, Alam M, Henriksson K, et al. Abnormal colonic microbial function in patients with rheumatoid arthritis. Scand J Rheumatol 1994;23:311-315.

Benno P, Alam M, Björklund N-E, et al. Host factors in susceptibility to arthritis induced with adjuvants (*M. tuberculosis*) or type II collagen in Agus rats. Scand J Lab Anim Sci 1996a;23,411-414.

Benno P, Sjöquist B, Midtvedt T. Excretion of olsalazine and sulphasalazine and their metabolites by germfree rats after a single oral administration. In: Hashimoto K, et al., eds. Germfree Life and its Ramifications. Xll ISG Publishing Committee, Shiozawa, Japan, 1996b;pp.451-456.

Benno P, Alam M, Midtvedt T, et al. Sulphasalazine, olsalazine and sulphapyridine induce mitogenic actions in the rat intestinal epithelium. APMIS 1997;105:717-722.

Benno P. Sulphasalazine, olsalazine and sulphapyridine influence microflora-associated characteristics in patients having ulcerative colitis and rheumatoid arthritis and also in experimental animals. "licentiatavhandling", Karolinska Institutet, Stockholm 1999.

Bjarnason I, Hayllar J, Macpherson J, et al. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 1993;104:1832-1847.

Björk J, Kleinau S, Midtvedt T, et al. Role of the bowel flora for development of immunity to hsp 65 and arthritis in three experimental models. Scand J Immunol 1994;40, 648-652.

Bradley SM, Neumann VC, Barr K, et al. Sequential study of bacterial antibody levels and faecal flora in rheumatoid arthritis patients taking sulphasalazine. Brit J Rheum 1993;32:683-688.

Bullock NR, Booth JC, Gibson GR. Comparative composition of bacteria in the human intestinal microflora during remission and active ulcerative colitis. Curr Issues Intest Microbiol 2004;5:59-64.

Burke DA & Axon ATR. Adhesive *Escherichia coli* in inflammatory bowel disease and infective diarrhoea. Brit Med J 1988;297:102-104.

Campieri M, Lanfranchi GA, Boschi S, et al. Topical administration of 5-aminosalicylic acid enemas in patients with ulcerative colitis. Studies on rectal absorption and excretion. Gut 1985;26:400-405.

Carlstedt-Duke B, Gustafsson BE, Midtvedt T. Clindamycin-induced alterations in intestinal Microflora-Associated Characteristics in rats. Scand J Gastroenterol 1985;20:92-98.

Cenac N, Coelho AM, Nguyen C, et al. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. Am J Pathol 2002; 161:1903-1915.

Collinder E. Intestinal Functions in Animals. Doctoral thesis, Karolinska Institutet, Stockholm, Sweden 2001.

Cooke EM. Faecal flora of patients with ulcerative colitis during treatment with salicylazosulphapyridine. Gut 1969;10:565-568.

Danese S, Sgambato A, Papa A, et al. Homocysteine triggers mucosal microvascular activation in inflammatory bowel disease. Am J Gastroenterol 2005;100:886-895.

Danielsson H & Gustafsson BE. On serum cholesterol levels and neutral fecal sterols in germfree rats. Bile acids and steroids 59. Arch Biochem Biophys 1959;83:482-485.

Das KM, Chowdhury JR, Zapp B, et al. Small bowel absorption of sulfasalazine and its hepatic metabolism in human beings, cats and rats. Gastroenterology 1979;77:280-284.

Dickinson RJ, Varian SA, Axon ATR, et al. Increased incidence of faecal coliforms with *in vitro* adhesive and invasive properties in patients with ulcerative colitis. Gut 1980;21:787-792.

Di Mario F, Glorioso S, Pagano R, et al. Histological modifications of gastric mucosa and functional correlations in rheumatoid arthritis. Curr Ther Res 1989;46:1153-1160.

Dubuquoy L, Rousseaux C, Thuru X, et al. PPARgamma as a new therapeutic target in inflammatory bowel diseases. Gut 2006;55:1341-1349.

Ebringer A, Khalafpour S, Wilson C. Rheumatoid arthritis and Proteus: a possible aetiological association. Rheumatol Int 1989;9:223-228.

Ekdahl C, Eberhardt K, Andersson SI, et al. Assessing disability in patients with rheumatoid arthritis: Use of a Swedish version of the Stanford Health Assessment Questionnaire. Scand J Rheumatol 1988;17:263-271.

Elson CO, Cong Y, Brandwein D, et al. Experimental models to study molecular mechanisms underlying intestinal inflammation. Ann N Y Acad Sci 1998;17:859,85-95.

Eyssen HJ, Parmentier GG, Compernolle FC, et al. Biohydrogenation of sterols by *Eubacterium* ATCC 21,408 - nova species. Europ J Biochem 1973;36:411-421.

Eyssen H & Parmentier G. Biohydrogenation of sterols and fatty acids by the intestinal microflora. Am J Clin Nutr 1974;27:1329-1340.

Fairbanks VF, Klee CG, Wisemann GA, et al. Measurement of blood volume and red cell mass: re-examination of ^{51}Cr and ^{125}I methods. Blood Cells Mol Dis 1996;22:169-186.

Fernández-Banares F, Hinojosa J, Sánchez-Lombrana JL, et al. Randomized clinical trial of Plantago ovata seeds (dietary fiber) as compared with mesalamine in maintaining remission in ulcerative colitis. Spanish Group for the Study of Crohn´s disease and ulcerative colitis (GETECCU). Am J Gastroenterol 1999;94:427-433.

Floch MH, Binder HJ, Filburn B, et al. The effect of bile acids on intestinal microflora. Am J Clin Nutr 1972;25:1418-1426.

Forchielli ML & Walker WA The role of gut-associated lymphoid tissues and mucosal defence. Brit J Nutr 2005; 93: Suppl 1:S41-48.

Freier TA, Beitz DC, Li L, et al. Characterization of *Eubacterium coprostanolignes* sp. nov., a cholesterol-reducing anaerobe. Int J Syst Bacteriol 1994;44:137-142.

Gaia E, Andriulli A, Tappero G, et al. Influence of sex and body size on pancreatic bicarbonate and enzyme output. J Clin Gastroenterol 1984;6:429-433.

Gérard P, Lepercq P, Leclerc M, et al. Bacteroides sp. strain D8, the first cholesterol-reducing bacterium isolated from human feces. Appl Environ Microbiol 2007;73:5742-5749.

Gran JT. The epidemiology of rheumatoid arthritis. In: Monograph Allergy, Schlumberger HD ed. Karger, Basel, 1987;162-196.

Greenfield C, Aguilar Ramirez JR, Pounder RE, et al. *Clostridium difficile* and inflammatory bowel disease. Gut 1983;24:713-717.

Grisham MB, Ware K, Marshall S et al. Prooxidant properties of 5-aminosalicylic acid. Possible mechanism for its adverse effects. Dig Dis Sci 1992;9:1383-1389.

Grisham MB. Oxidants and free radicals in inflammatory bowel disease. Lancet 1994;344:859-861.

Gustafsson BE. Vitamin K deficiency in germfree rats. Ann NY Acad Sci 1959;78:166-173.

Gustafsson BE & Norin KE. Development of germfree animal characteristics in conventional rats by antibiotics. Acta Path Microbiol Scand Sect B 1977;85:1-8.

Gustafsson JÅ & Werner B. Fecal sterols in infants. Acta Physiol Scand 1968;73:305-310.

Hartley MG, Hudson MJ, Swarbrick ET, et al. The rectal mucosa-associated microflora in patients with ulcerative colitis. J Med Microbiol 1992;36:96-103.

Hawrelak JA & Myers SP. The causes of intestinal dysbiosis: A review. Altern Med Rev 2004;9:180-197.

Hazenberg MP, Bakker M, Both-Patoir HC, et al. Effect of sulphasalazine on the human intestinal flora. J Appl Bacteriol 1982;52:103-107.

Hazenberg MP, Klasen IS, Kool J, et al. Are intestinal bacteria involved in the etiology of rheumatoid arthritis? APMIS 1992;100:1-9.

Henriksson K, Uvnäs-Moberg K, Nord C-E, et al. Gastrin, gastric acid secretion, and gastric microflora in patients with rheumatoid arthritis. Ann Rheum Dis 1986;45:475-483.

Henriksson K, Blomquist L, Nord C-E, et al. Small intestinal bacterial overgrowth in patients with rheumatoid arthritis. Ann Rheum Dis 1993;52:503-510.

Hill MJ. The effect of some factors on the faecal concentration of acid sterols, neutral steroids and urobilins. Pathology 1971;104:239-245.

Hill MJ & Aries VC. Faecal steroid composition and its relationship to cancer of the large bowel. J Pathol 1971;104:129-139.

Holzapfel WH, Haberer P, Snel J, et al. Overview of gut flora and probiotics. Int J Food Microbiol 1998;41:85-101.

Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 2002;22:283-307.

Huseby E, Hellström PM, Sundler F, et al. Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. Am J Physiol Gastrointest Liver Physiol 2001;280:368-380.

Jawetz E, Melnick JL, Adelberg EA, eds. Cell structure. In: Review of Medical Microbiology, 7th edition. Appleton & Lange, Norwalk, Connecticut, Los Altos, California 1987;pp.6-29.

Johnson-Henry KC, Hagen KE, Gordonpour M, et al. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* 0157:H7 adhesion to epithelial cells. Cell Microbiol 2007;9:356-367.

Kanerud L, Scheynius A, Nord CE, et al. Effect of sulphasalazine on gastrointestinal microflora and on mucosal heat shock protein expression in patients with rheumatoid arthritis. Brit J Rheumatol 1994;33:1039-1048.

Karlsson K-A. Animal glycosphingolipids as membrane attachment sites for bacteria. Annu Rev Biochem 1989;58:309-350.

Karlsson K-A, Ångström J, Teneberg S. Characteristics of the recognition of host cell carbohydrates by viruses and bacteria. In: Wadström T et al., eds. Molecular Pathogenesis of Gastrointestinal Infections. New York, U.S.A: Plenum Press, 1991:pp.9-21.

Katouli M, Erhardt-Bennet AS, Kühn I, et al. Metabolic capacity and pathogenic properties of the intestinal coliforms in patients with ulcerative colitis. Microb Ecol Health Dis 1992;5:245-255.

Keighley MRB, Arabi Y, Dimock F, et al. Influence of inflammatory bowel disease on intestinal flora. Gut 1978;19:1099-1104.

Kellog TF & Wostmann BS. The effect of carbohydrate digestibility on fecal steroids. Biochem Biophys Acta 1966;125:617-619.

Kellogg TF. On the site of the microbiological reduction of cholesterol to coprostanol in the rat. Lipids 1973;8:86-89.

Kohashi O, Kohashi Y, Takahashi T, et al. Reverse effect of Gram-positive bacteria vs. Gram-negative bacteria on adjuvant-induced arthritis in germfree rats. Microbiol Immunol 1985;29:487-497.

Korpela JT, Hämäläinen E, Adlercreutz H. Effect of oxytetracycline on bacterial intestinal metabolism of neutral sterols and on serum lipids. Scand J Gastroenterol 1984;19:401-404.

Korpela JT & Adlercreutz H. Fecal neutral sterols in omnivorous and vegetarian women. Scand J Gastroenterol 1985; 20:1180-1184.

Kruis W, Fric P, Pokrotnieks J, et al. Maintaining remission of ulcerative colitis with the probiotic Escheria coli Nissle 1917 is as effective as with standard mesalazine. Gut 2004;53:1617-1623.

Laugier R, Bernard JP, Berthezene P, et al. Changes in pancreatic exocrine secretion with age: pancreatic exocrine secretion does decrease in the elderly. Digestion 1991;50:202-211.

Lauritsen K, Laursen LS, Bukhave K, et al. Effects of topical 5-aminosalicylic acid and prednisolone on prostaglandin E2 and leukotriene B4 levels determined by equilibrium in vivo dialysis of rectum in relapsing ulcerative colitis. Gastroenterology 1986;91:837-844.

Ligumsky M, Karmeli F, Sharon P, et al. Enhanced thromboxane A_2 and prostacyclin production by cultured rectal mucosa in ulcerative colitis and its inhibition by steroids and sulfasalazine. Gastroenterology 1981;81:444-449.

Li L, Batt SM, Wannemuehler M, et al. Effect of feeding of a cholesterol-reducing bacterium, *Eubacterium coprostanolignes*, to germ-free mice. Lab Anim Sci 1998;48:253-255.

Lipkin M, Reddy BS, Weisburger J, et al. Nondegradation of fecal cholesterol in subjects at high risk for cancer of the large intestine. J Clin Invest 1981;67:304-307.

Marchbank T, Freeman TC, Playford RJ. Human pancreatic secretory trypsin inhibitor. Digestion 1998;59:167-174.

Marcolongo R, Bayeli PF, Montagnani M. Gastrointestinal involvement in rheumatoid arthritis: a biopsy study. J Rheumatol 1979;6:163-173.

Mattern T, Thanhauser A, Reiling N, et al. Endotoxin and lipid A stimulate proliferation of human T cells in the presence of autologous monocytes. J Immunol 1994;153:2996-3004.

McConkey B, Amos RS, Durham S, et al. Sulphasalazine in rheumatoid arthritis. Brit Med J 1980;280:442-444.

McDonagh J, Gray J, Sykes H, et al. Anti-*Proteus* antibodies and *Proteus* organisms in rheumatoid arthritis: a clinical study. Brit J Rheumatol 1994;33:32-35.

Meier R, Beglinger C, Dederding JP, et al. Influence of age, gender, hormonal status and smoking habits on colonic transit time. Neurogastroenterol Mot 1995;7:235-238.

Midtvedt T. Microbial bile acid transformation. Am J Clin Nutr 1974;27:1341-1347.

Midtvedt T & Frederichsen P. Influence of antibiotics on microbial intestinal transformation of cholesterol to coprostanol in man. Scand J Gastroenterol 1977;12:669-672.

Midtvedt T & Gustafsson BE. Microbial conversion of bilirubin to urobilins in vitro and in vivo. Acta Path Microbiol Scand Sect B 1981;89:57-60.

Midtvedt T. Microflora-associated characteristcs (MACs) and germfree animal characteristics (GACs) in man and animal. Microecol Therapy 1985a;15:295-302.

Midtvedt T, Björneklett A, Carlstedt-Duke B, et al. The influence of antibiotics upon microflora-associated characteristics in man and mammals. In: Wostmann BS, et al., eds. Germ-free Research, Microflora Control and its Application to the Biomedical Sciences. Alan R. Liss, New York, 1985b, pp.241-244.

Midtvedt T. Metabolism of endogenous substances. In: Clercq De, ed. Frontiers in microbiology. From antibiotics to AIDS. Dordrecht/Boston/Lancaster: Martinus Nijhoff Publishers, 1987:pp.79-87.

Midtvedt AC, Carlstedt-Duke B, Norin KE, et al. Development of five metabolic activities associated with the intestinal microflora of healthy infants. J Pediatr Gastroenterol Nutr 1988;7:559-567.

Midtvedt T. Influence of antibiotics on biochemical intestinal microflora-associated characteristics in man and animals. In: Gillesen, et al. eds. The Influence of Antibiotics on the Host-Parasite Relationship III. Berlin, Heidelberg: Springer Verlag, 1989:pp.209-215.

Midtvedt T, Lingaas E, Carlstedt-Duke B, et al. Intestinal microbial conversion of cholesterol to coprostanol in man. APMIS 1990;98:839-844.

Midtvedt A-C & Midtvedt T. Conversion of cholesterol to coprostanol by the intestinal microflora during the first two years of life. J Ped Gastroenterol Nutr 1993;17:161-168.

Midtvedt T. Microbial functional activities. In: Hanson LÅ & Yolken RH, eds. Probiotics, Other Nutritional Factors, and Intestinal Microflora. Nestec, Ltd., and Lippincott-Raven Publishers, Pennsylvania, 1999; pp.79-96.

Midtvedt T. Nitrogen and gastrointestinal microbiota – Comparative studies in germfree and conventional animals. Microb Ecol Health Dis 2006;18:90.

Miller JM & O´Hara CM. Substrate utilization systems for the identification of bacteria and yeasts. In: Murray PR, et al., eds. Manual of Clinical Microbiology. 6th edition, American Society for Microbiology, Washington 1995;pp.103-109.

Mital BK & Garg SK. Anticarcinogenic, hypocholestrolemic and antagonistic activities of *Lactobacillus acidophilus*. Crit Rev Microbiol 1995;21:175-214.

Mukherjee S, Partch CL, Lehotzky RE, et al. Regulation of C-type lecitin antimicrobial activity by a flexible N-terminal prosegment. J Biol Chem 2009;284:4881-4888.

Mäkivuokko H & Nurminen P. In vitro methods to model the gastrointestinal tract. In: Ouwehand AC & Vaughan EE, eds. Gastrointestinal Microbiology. Taylor & Francis, New York 2006;pp.237-252.

Neumann VC, Shinebaum R, Cooke EM, et al. Effects of sulphasalazine on faecal flora in patients with rheumatoid arthritis: a comparison with penicillamine. Brit J Rheumatol 1987;26:334-337.

Neutra MR & Forstner JF. Gastrointestinal mucus: synthesis, secretion and function. In: Johnson LR, ed. Physiology of the Gastrointestinal Tract. 2nd ed. Raven Press, New York 1987;pp.975-1009.

Nielsen OH & Ahnfelt-Rønne I. Involvement of oxygen-derived free radicals in the pathogenesis of chronic inflammatory bowel disease. Klin Wochenschr 1991;69:995-1000.

Nordenvall B, Broström O, Berglund M, et al. Incidence of ulcerative colitis in Stockholm County 1955-1979. Scand J Gastroenterol 1985;20:783-790.

Norin KE, Gustafsson BE, Midtvedt T. Strain differences in faecal tryptic activity of germfree and conventional rats. Lab Anim 1986a;20:67-69.

Norin, KE, Midtvedt T, Gustafsson BE. Influence of intestinal microflora on the tryptic activity during lactation in rats. Lab Ani Sci 1986b; 20:234-237.

Norin KE, Carlstedt-Duke B, Höverstad T, et al. Faecal tryptic activity in humans: influence of antibiotics on microbial intestinal degradation. Microb Ecol Health Dis 1988;1:65-68.

Norin KE. The normal microflora and intestinal enzymes. In: Grubb R, et al., eds. The Regulatory and Protective Role of the Normal Microflora. London, Great Britain; The Macmillan Press LDT,1989:pp.219-237.

Norin KE, Gustafsson J-Å, Johansson G, et al. Effects of lacto-vegetarian diet on some microflora-associated characteristics: a long term study. Microb Ecol Health Dis 1998;10:79-84.

Norin E, Jernberg C, Nilsson H-O, et al. Studies of the intestinal microflora by traditional, functional and molecular techniques. In: Ljung Å, et al. eds. Lactobacillus Molecular Biology from Genomics to Probiotics. Norfolk, UK; Caister Academic Press, 2009: pp. 83-94.

42

Norkost 1993-94 & 1997. Rapport nr 3/1999. Statens råd for ernaering og fysisk aktivitet. Oslo, 1999.

O´Farrelly C, Marten D, Melcher D, et al. Association between villous atrophy in rheumatoid arthritis and a rheumatoid factor and gliadin-specific IgG. Lancet 1988;8:819-822.

Olhagen B & Månsson I. Intestinal *Clostridium perfringens* in rheumatoid arthritis and other collagen diseases. Acta Med Scand 1968;184:395-402.

Osman N, Adawi D, Molin G, et al. *Bifidobacterium infantis* strains with and without a combination of oligofructose and inulin (OFI) attenuate inflammation in DSS-induced colitis in rats. BMC Gastroenterol 2006;28;6:31**.**

Parkkila S, Parkkila A-K, Juvonen T, et al. Distribution of the carbonic anhydrase isoenzyme I, II, and VI in the human alimentary tract. Gut 1994;35:646-650.

Peltonen R, Kjeldsen-Kragh J, Haugen M, et al. Changes of faecal flora in rheumatoid arthritis during fasting and one-year vegetarian diet. Brit J Rheumatol 1994;33:638-643.

Peppercorn M & Goldman P. The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. J Pharmacol Exp Ther 1972;181:555-562.

Peuchant E, Salles C, Jensen R. Relationship between fecal neutral steroid concentrations and malignancy in colon cells. Cancer 1987;60:994-999.

Playford RJ, Hanby AM, Patel K, et al. Influence of inflammatory bowel disease on the distribution and concentration of pancreatic secretory trypsin inhibitor within the colon. Am J Pathol 1995;146:31-316.

Puleston J, Cooper M, Murch S, et al. A distinct subset of chemokines dominates the mucosal chemokine response in inflammatory bowel disease. Aliment Pharmacol Ther 2005; 21:109-120.

Pullar T, Hunter JA, Capell HA. Which component of sulphasalazine is active in rheumatoid arthritis? Br Med J 1985;290:1535-1538.

Raithel M, Winterkamp S, Pacurar A, et al. Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. Scand J Gastroenterol 2001;36:174-179.

Ramare F, Hautefort I, Verhe F, et al. Inactivation of tryptic activity by a human-derived strain of *Bacteroides distasonis* in the large intestines of gnotobiotic rats and mice. Appl Environ Microbiol 1996;62:1434-1436.

Reddy BS & Wynder EL. Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. J Natl Cancer Inst 1973;50:1437-1442.

Reddy BS, Martin CW, Wynder EL. Fecal bile acids and cholesterol metabolites of patients with ulcerative colitis, a high-risk group for development of colon cancer. Cancer Res 1977;37:1697-1701.

Reddy BS, Engle A, Simi B, et al. Effect of dietary fiber on colonic bacterial enzymes and bile acids in relation to colon cancer. Gastroenterology 1992;102:1475-1482.

Rembacken BJ, Snelling AM, Hawkey P et al. Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 1999; 354;635-639.

Ren D, Li L, Schwabacher AW, et al. Mechanism of cholesterol reduction to coprostanol by Eubacterium coprostanoligenes ATCC 51222. Steroids 1996;61:33-40.

Riksmaten 1997-98. Kostvanor och näringsintag i Sverige, metod- och resultatanalys. Livsmedelsverket. Tryck-jouren, Uppsala, Sweden, 2002.

Roediger WE. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. Gut 1980;21:793-798.

Rogers P, Hassan J, Bresnihan B, Feighery C, et al. Antibodies to Proteus in rheumatoid arthritis. Br J Rheumatol 1988;27(suppl 2):90-94.

Rød T & Midtvedt T. Origin of intestinal ß-glucoronidase in germfree, monocontaminated and conventional rats. Acta Path Microbiol Scand Sect B 1977;85:271-276.

Sadzikowski MR, Sperry JF, Wilkins TD. Cholesterol-reducing bacterium from human feces. Appl Environ Microbiol 1977;34:355-362.

Salyers AA, Sperry JF, Wilkins TD, et al. Neutral steroid concentrations in the faeces of North American white and South African black populations at different risks for cancer of the colon. S Afr Med J 1977;51:823-827.

Samanci N, Ozdem S, Akbas H, et al. Diagnostic value and clinical significance of anti-CCP in patients with advanced rheumatoid arthritis. J Natl Med Assoc 2005; 97:1120-1126.

Sandberg-Gertzén H, Kjellander J, Sundberg-Gillå B, et al. In vitro effects of sulphasalazine, azodisal sodium, and their metabolites on *Clostridium difficile* and some other faecal bacteria. Scand J Gastroenterol 1985;20:607-612.

Sapico FL, Emori H, Smith LDS, et al. Absence of relationship of fecal *Clostridium perfringens* to rheumatoid arthritis and rheumatoid variants. J Infect Dis 1973;128:559-562.

Saxerholt H. Intestinal Bilirubin Metabolism, Doctoral thesis, Karolinska Institutet, Stockholm, Sweden 1990.

Schröder H. Azo reduction of salicyl-azo-sulphapyridine in germfree and conventional rats. Xenobiotica 1973;3:225-231.

Schumacher G. First attack of inflammatory bowel disease and infectious colitis. A clinical, histological and microbiological study with special reference to early diagnosis. Scand J Gastroenterol 1993;28,suppl 198.

The Scottish health survey 1998. The National Centre for Social Research and Department of Epidemiology & Public Health. http://www.show.scot.nhs.uk/scottishhealthsurvey/sh800.html

Shinebaum R, Neumann VC, Cooke EM, et al. Comparison of faecal florae in patients with rheumatoid arthritis and controls. Br J Rheum 1987;26:329-333.

Sobko T. Influence of the microflora on gastrointestinal nitric oxide (NO) generation: studies in newborn infants and germ-free animals. Microb Ecol Health Dis 2006;18:91.

Stenson WF & Lobos E. Sulfasalazine inhibits the synthesis of chemotactic lipids by neutrophils. J Clin Invest 1982;69:494-497.

Stern NJ, Svetoch EA, Eruslanov BV, et al. Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrob Agents Chemother 2006;50:3111-3116.

Stokkers PC & Hommes DW. New cytokine therapeutics for inflammatory bowel disease. Cytokine 2004;28:167-173.

Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nature Publishing Group www.nature.com/reviews/drugdisc 2008;7:168-181.

Svartz N. The primary cause of rheumatoid arthritis is an infection - the infectious agents exist in milk. Acta Med Scand 1972;192:231-239.

Tysk C & Järnerot G. Ulcerative proctocolitis in Örebro, Sweden. A retrospective epidemiologic study, 1963-1987. Scand J Gastroenterol 1992;27:945-950.

van der Wiel-Korstanje JAA & Winkler KC. The faecal flora in ulcerative colitis. J Med Microbiol 1975;8:491-501.

van Hees PA, van Elferen LW, van Rossum JM, et al. Hemolysis during salicylazosulfapyridine therapy. Am J Gastroenterol 1978; 70:501-505.

Verspaget HW, Aparicio-Pagés MN, Verver S, et al. Influence of sulphasalazine and mesalazine on cellular and biochemical oxygen metabolite production. Effect of in vivo administration and an in vitro analysis. Scand J Gastroenterol 1991;26:779-786.

Washington JA. Principles of diagnosis. In: Baron S, ed. Medical Microbiology, 3rd ed. Churchill Livingstone Inc.,New York 1991; pp.167-177.

Wehkamp J & Stange EF. Paneth cells and the innate immune response. Curr Opin Gastroenterol 2006;22:644-650.

Weiner SR, Clarke J, Taggart NA, et al. Rheumatic manifestations of inflammatory bowel disease. Semin Arthritis Rheum 1991;20:353-366.

Wells WW & Cooper SB. Coprostanol formation. Part II: The opposing action of dietary lactose and calcium on cholesterol absorption. Arch Biochem Biophys 1958;75:273-279.

West B, Lendrum R, Hill MJ, et al. Effects of sulphasalazine (Salazopyrin) on faecal flora in patients with inflammatory bowel disease. Gut 1974;15:960-965.

Wilkins TD & Hackman AS. Two patterns of neutral steroid conversion in the feces of normal North Americans. Cancer Res 1974;34:2250-2254.

Vítek L, Zelenka J, Zadinová M, et al. The impact of intestinal microflora on serum bilirubin levels. J Hepatol 2005:42;238-243.

Woods DD. The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide. Br J Exp Pathol 1940;21:74-90.

Wostmann BS, Larkin C, Moriarty A, et al. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Lab Anim Sci 1983;33:46-50.

Yoshida N, Isozaki Y, Takagi T, et al. Review article: anti-tryptase therapy in inflammatory bowel disease. Aliment Pharmacol Ther 2006;24:249-255.

Zvaifler NJ. Clinical aspects of rheumatoid arthritis. In: Firestein GS, Panayi GS, Wollheim FA, eds. Rheumatoid Arthritis. 2nd edition. Oxford University Press, New York 2006.pp.245-258.